Inositol phosphate-mediated iron transport in Pseudomonas aeruginosa

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INOSITOL PHOSPHATE-MEDIATED IRON TRANSPORT IN *PSEUDOMONAS AERUGINOSA*

submitted by Peter Hanson Hirst

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To Mum and Dad

Thesis Summary

The aim of this work was to elucidate the mechanisms underlying the siderophore activity of *myo*-inositol hexakisphosphate (*myo*-$InsP_6$). *myo*-$InsP_6$ is found in the natural environment of *P. aeruginosa* and in many, if not all, plant and animal cells.

Using a range of lower inositol phosphates, it was possible to identify key structural motifs responsible for inositol phosphate-mediated iron uptake. Using the hydroxyl radical assay, the 1,2,3 trisphosphate motif appears to provide the optimum conformation for interaction with ferric iron. However, this conformation is associated with a particularly low ability to mediate iron transport in *P. aeruginosa*. Conversely, *myo*-Ins(1,4,5)P_3 appears to have a lower ability to interact with iron and is associated with a larger degree of iron transport. This inverse relationship was attributed to the differences in abilities to yield iron to a putative carrier system. Models are proposed to illustrate ferri-inositol phosphate interactions.

In attempting to identify mechanisms responsible for iron release from inositol phosphates, conformational mobility of the inositol ring was not regarded as essential. However, *P. aeruginosa* reductase activity was capable of removing iron from the range of inositol phosphates tested. With respect to the bacterial mechanisms involved in *myo*-$InsP_6$-mediated iron transport, the integrity of the outer membrane and the dependence upon active transport systems were noted as key factors. Specific binding of [^{3}H]-*myo*-$InsP_6$ to the outer membrane was demonstrated although this did not appear to be *via* a specific outer membrane protein. Finally, it was not possible to isolate a mutant unable to use ferri-*myo*-$InsP_6$ as an iron source, although a model was proposed to explain this alternative mechanism of iron acquisition in *P. aeruginosa*.

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Abbreviations

°C	degrees centigrade
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CF	cystic fibrosis
Da	dalton(s)
DNA	deoxy-ribonucleic acid
EDDHA	ethylene diamine di(*o*-hydroxyphenyl) acetic acid
EDTA	ethylene diamine tetra-acetic acid
Fe(II)	ferrous iron
Fe(III)	ferric iron
g	gramme(s)/gauge
h	hour(s)
Hg^{15}	mercury (II) chloride at 15 µg/ml
InsPx	inositol phosphate
kb	kilobase
kDa	kilodalton(s)
Km^{250}	kanamycin at 250 µg/ml
l	litre(s)
LA	Luria agar
LB	Luria broth
M	mole(s) per litre
MBq	mega bequerel(s)
µg	microgramme(s)
µl	microlitre(s)
µm	micrometre(s)
µM	micromole(s) per litre
met	methionine
mg	milligramme(s)
min	minute(s)

ml	millilitre(s)
mm	millimetre(s)
mM	millimole(s) per litre
MOPS	3-[N-Morpholino] propane-sulphonic acid
NA	nutrient agar
NAD	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form.
nm	nanometre(s)
nM	nanomole(s) per litre
OD_x	optical density at x nanometres
OM	outer membrane
OMP	outer membrane protein
Opr	*Pseudomonas* outer membrane protein
Pch	pyochelin
pmol	picomole
Pvd	pyoverdine
rpm	revolutions per minute
s	second (s)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Str^{750}	streptomycin at 750 μg/ml
Tn	transposon
Tris	tris (hydroxymethyl) amino ethane
V	volt(s)
$^{v}/_{v}$	volume by volume
$^{w}/_{v}$	weight by volume

Chapter 1

Introduction

1.1. The Biological Importance of Iron

Iron is the fourth most abundant element on earth and impacts significantly on many biological systems. It is the second most abundant metal after aluminium (reviewed by Neilands, 1991). It is an essential component in cytochromes, hydroperoxidases, in iron-sulphur proteins and ribonucleotide reductase and most life-forms cannot exist in the absolute absence of iron (Neilands, 1991).

Iron has unusual chemistry in that it can exist as either the Fe(III) (ferric) or the Fe(II) (ferrous) oxidation state. However, the majority of iron is present as Fe(III). Fe(II) is relatively soluble and it is possible to obtain a 100 mM solution at neutral pH. However, because of the formation of insoluble oxyhydroxide polymers of the general composition FeOOH e.g. goethite and hematite, the greatest concentration of Fe (III) possible in the same conditions is approximately 10^{-18} M (Neilands, 1991). To sustain bacterial growth, Fe(III) must be present between 10^{-8} to 10^{-6} M, hence many bacteria have developed iron-uptake systems that overcome the insolubility of this important element. Such systems permit iron acquisition and its subsequent uptake into the bacterium.

In vivo iron availability is also very low despite large amounts being present. The human body contains 4 to 5g of iron (Bagg and Neilands, 1987b), approximately 75% of which is bound in haemoglobin. Much of the remainder is stored in the liver in ferritin resulting in only trace amounts being available for enzyme and transport functions. The human body is remarkably efficient in its use and internal cycling of iron. Iron from old erythrocytes is salvaged, stored temporarily in ferritin and then used in the synthesis of new haemoglobin. The cycle is so efficient that only 1 mg of iron is required each day to maintain homeostasis. This translates to a daily dietary requirement of approximately 10 mg (reviewed by Neilands, 1991).

Most extracellular iron is found in body fluids such as plasma and mucosal secretions bound to the high affinity iron-binding glycoproteins transferrin and lactoferrin (Griffiths, 1991). Transferrin is the major plasma iron-binding glycoprotein and lactoferrin is found primarily in mucosal secretions.

During infection, the host is able to reduce significantly the amount of free iron available by two processes. One process involves the release of lactoferrin from polymorphonucleocyte (PMN) leucocytes which, having a high affinity for iron, removes iron from serum transferrin (Webster *et al.*, 1980). Iron-lactoferrin complexes are thought to be internalised by macrophages and eliminated by the reticuloendothelial system (van Snick *et al.*, 1974; Klempner *et al.*, 1978). An alternative method involves iron bound to transferrin being transferred to intracellular storage in ferritin molecules (Konijn and Hershko, 1989). This non-

specific mechanism forms a first line defence mechanism against invading pathogens.

The level of iron within the cell must be controlled precisely since an excess leads to the formation of damaging free radicals. It is the versatility of iron, with its two stable valencies, that confers considerable potential for a range of oxidation/reduction reactions and chemical reactivities. One particular problem is that iron is a central component in Haber-Weiss-Fenton chemistry.

Iron catalyses the formation of highly reactive hydroxyl free radicals that react with many biological molecules . In particular, hydroxyl free radicals damage DNA and perturb membranes by peroxidation of lipids (Halliwell and Gutteridge, 1984). Haber-Weiss-Fenton chemistry is summarised as follows:-

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Precise regulation is therefore essential to ensure that the intracellular iron concentration is maintained at a level suitable for cell homeostasis and yet not cause toxicity. In iron-replete conditions, iron is stored in the intracellular storage compound ferritin and cellular iron-uptake *via* the transferrin receptor is limited. Conversely, in iron-deplete conditons, ferritin synthesis is reduced and the cell directs synthesis of the transferrin receptor to acquire iron. Iron levels in mammalian

cells are maintained, in part, by the reciprocal control of the transferrin receptor and ferritin mRNA *via* a cytosolic protein termed the iron-regulatory factor (IRF) (Mullner *et al.*, 1989). All ferritin mRNAs have an iron-responsive element (IRE) located in their 5' untranslated regions (Casey *et al.*, 1988) and a similar structure is found in the 3' untranslated regions of TfR (transferrin receptor) mRNAs. Regulation occurs as a result of IRF binding the IREs of the relevant mRNAs. In iron-deplete conditions, IRF binds to the IRE of TfR mRNA, protecting it from nuclease digestion hence enhancing TfR expression. This leads to increased iron-uptake into the cell. In addition, IRF binds to the IRE of ferritin mRNA, but in this case blocks ferritin production. However, in iron-replete conditions, IRF dissociates from TfR mRNA resulting in its degradation leading to reduced TfR production. IRF binds less tightly to ferritin mRNA resulting in increased expression with a subsequent increase in ferritin production. Consequently, excess iron becomes bound to ferritin in a form that cannot take part in Haber-Weiss chemistry (Ward *et al.*, 1994).

Pseudomonas aeruginosa, in common with many other bacteria, produces a ferritin-like compound, termed bacterioferritin, for intracellular iron storage (Moore *et al.*, 1986). The three-dimensional structures of the bacterioferritins are similar to animal ferritins and most contain 3 to 12 haem groups in 24 subunits of 17 to 19 kDa. In addition, bacterioferritins contain 8 to 10% by weight of non-haem iron (Moore *et* al., 1994).

1.2. The Response of Gram-negative Bacteria to Iron Limitation

In response to iron-deplete conditions, many Gram-negative bacteria derepress high-affinity iron-uptake mechanisms based on the production of siderophores which scavenge environmental iron and transport it back to the bacterium. In addition, many Gram-negative bacteria produce exoproducts capable of inflicting considerable damage to host tissue. Exoproduct-mediated cellular damage results in the release of intracellular iron which also becomes available for the bacterium.

Siderophores have extremely high affinities towards ferric iron and enable solubilisation and transport of the metal into the bacterial cell *via* proteins located in the outer membrane. *In vivo* siderophore production has been confirmed for several pathogens. These include *P. aeruginosa*, whose siderophores have been observed in the sputa of cystic fibrosis patients (Haas *et al.*, 1991a) and *Vibrio cholerae* which releases the siderophore vibriobactin (Griffiths *et al.*, 1984) to collect iron released as a result of haemolysin-mediated cell damage (Honda and Finkelstein, 1979). In addition, schizokinen, a citrate based siderophore produced by *Bacillus* and *Anabaena* spp, has been purified directly from soil samples (Akers, 1983).

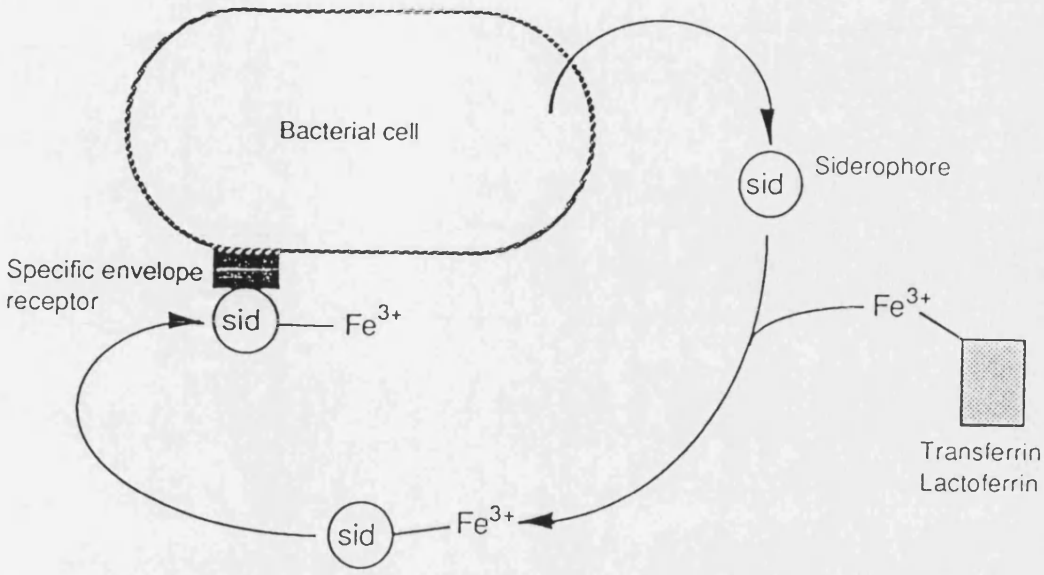


Figure 1.1. Bacterial siderophore-mediated iron-uptake where sid = siderophore (adapted from Griffiths, 1987).

Other human pathogens have developed methods for removing iron directly from transferrin and lactoferrin. *Neisseria gonnorhoeae* and *Neisseria meningitidis* have outer membrane protein receptors that remove iron directly from host glycoproteins. The receptors are specific for transferrin or lactoferrin, since neither glycoprotein is capable of blocking the binding of the other. By binding the relevant glycoprotein, the organism is able to scavenge sufficient iron for growth (reviewed by Wooldridge and Williams, 1993).

1.3. The Clinical Importance of *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative opportunist pathogen of considerable clinical importance (Hancock, 1986) It is carried by many people and is ranked alongside *Enterococcus faecalis* and *Escherichia coli* as being one of the major causes of

nosocomial infections (Schaberg *et al.*, 1991). However, *P. aeruginosa* rarely causes infections in healthy people and, in most cases, the disease process begins with some alteration or circumvention of the normal host defences (Pollack, 1990). *P. aeruginosa* is associated with several life-threatening infections in patients already suffering debilitating illness. These include the immunocompromised, following transplant surgery or as a result of leukaemia, and patients suffering from severe burns, cancer and diabetes mellitus. It is also the leading cause of mortality in cystic fibrosis (CF) patients (Koch and Hoiby, 1993).

1.3.1. *Pseudomonas aeruginosa* in Cystic Fibrosis

CF is the most common autosomal recessive genetic disease amongst Caucasians affecting approximately 1 in 2500 live births (Collins, 1992). Mutations causing CF occur in the gene encoding a cAMP-regulated transmembrane chloride channel called the cystic fibrosis transmembrane regulator (CFTR) protein. In the lung, deficiencies in CFTR impair chloride and water secretion by the respiratory epithelium, resulting in the formation of thick dehydrated mucus that is difficult to clear. This seems to provide some micro-organisms with a favourable growth environment. The most common infective agents are *P. aeruginosa*, *Burkolderia* (*Pseudomonas*) *cepacia*, and *Staphylococcus aureus*, although *Haemophilus influenzae*, non-tuberculous mycobacteria and *Aspergillus fumigatus* have also been isolated (Deretic *et al.*, 1995). Of these, *P. aeruginosa* is the most prevalent with over 80% of patients eventually becoming infected (Koch and Hoiby, 1993). Once established, *P. aeruginosa* is difficult to control particularly since it is inherently resistant to many antibiotics. Moreover, this is exacerbated by conversion to a

mucoid phenotype where the exopolysaccharide alginate is overproduced. Death resulting from chronic infection is usually a result of airway damage that impairs gas exchange that ultimately causes a fatal deterioration in respiratory function.

1.4. Siderophore Systems of *Pseudomonas aeruginosa*

P. aeruginosa responds to iron-limiting conditions by derepressing high-affinity iron-uptake systems based on two unrelated siderophores, pyochelin and pyoverdine. The combination of pyoverdine and pyochelin increases the ability of *P. aeruginosa* to grow in the presence of human serum and transferrin (Cox, 1982; Ankenbauer *et al.*, 1985).

A recent study by Meyer *et al.*, (1996) indicates that pyoverdine is essential for the virulence of *P. aeruginosa*. Using a mouse model, pyoverdine-deficient mutants were less virulent than the pyoverdine-producing equivalent which caused eventual death. Virulence of the pyoverdine-deficient strain was restored when purified pyoverdine originating from the wild-type strain was added to the site of infection. Competition experiments, demonstrating the ability of pyoverdine to remove iron from ferritransferrin *in vitro*, supported these findings.

Mucoid strains of *P. aeruginosa* isolated from the lungs of infected CF patients produce pyoverdine and pyochelin in a manner similar to their non-mucoid counterparts (Haas *et al.*, 1991b). Pyoverdine has a much higher affinity for Fe(III) than pyochelin ($K = 10^{32}\ M^{-1}$ and $2.5 \times 10^{5}\ M^{-1}$, respectively), and is regarded as the

more important siderophore (Cox and Graham, 1979; Ankenbauer *et al.*, 1985; Wendenbaum *et al.*, 1983).

1.4.1. Pyochelin

Pyochelin was first isolated by Cox and Graham (1979) from ethyl acetate extracts of *P. aeruginosa* culture supernatants. It is synthesised during active growth in culture media containing limited amounts of $FeCl_3$. When added to iron-deficient cultures, pyochelin promotes bacterial growth and can reverse growth inhibition by the iron-chelator ethylenediamine di-(*o*-hydoxyphenylacetic acid) (EDDHA). Pyochelin production is not unique to *P. aeruginosa* and is also produced by *Burkholderia* (*Pseudomonas*) *cepacia* and *Pseudomonas fluorescens* (Cuppels *et al.*, 1987; Sokol, 1986).

Pyochelin is structurally unique and has neither hydroxamate nor catecholic-chelating groups (Ankenbauer and Quan, 1994). The pyochelin molecule consists of a salicyl ring bonded to a thiazoline ring which is itself bonded to a N-methylthiazolidine ring (Cox *et al.*, 1981) and has been assigned the nomenclature 2-[2-(*o*-hydroxyphenyl)-2-thiazoline-4-yl]-3-methyl-4-thiazolidine carboxylic acid. The unusual combination of thiazoline and thiazolidine heterocyclic rings in pyochelin has not been observed in other siderophores. Only anguibactin, from *Vibrio anguillarium* possesses a thiazoline ring (Jalal *et al.*, 1989). However, unlike pyochelin, anguibactin also has both hydroxamate and catecholate-chelating groups.

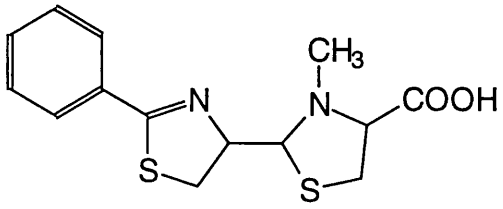


Figure 1.2. The structure of pyochelin (Cox *et al.*, 1981).

1.4.2. Pyochelin-associated Outer Membrane Proteins

The receptor for ferripyochelin was initially suggested to be a weakly iron repressible 14 kDa outer membrane protein (Sokol and Woods, 1986). Antibodies raised against the 14 kDa receptor were able to reduce, ferripyochelin uptake (Sokol and Woods, 1986) and a mutant deficient in surface expression of this protein was devoid of ferripyochelin uptake (Sokol, 1987).

Heinrichs *et al.* (1991) described a 75 kDa OMP, strongly derepressed under iron-deficient conditions, that was involved with pyochelin-mediated iron uptake, suggesting that *P. aeruginosa* may have two ferripyochelin uptake systems. A mutant deficient in expression of the 75 kDa protein still showed ferripyochelin uptake and expressed the 14 kDa OMP. Further studies illustrated that the uptake system utilising the high molecular weight OMP operated at iron concentrations 5-10 fold lower than those required for uptake *via* the 14 kDa system. These apparent differences in affinity suggest that the proposed low affinity system functions during marginal iron-limitation with the high affinity uptake system coming into effect as iron limitation becomes more severe. In addition, the 75 kDa OMP is expressed only in late log or early stationary phase cells, which in batch culture at least, are expected

to be more iron-limited than early log phase cells. Early log phase cells appear to transport ferripyochelin *via* the less iron-repressible 14 kDa protein.

Ankenbauer and Quan (1994) isolated and cloned the 75 kDa ferripyochelin receptor, FptA. It has strong homology with hydroxamate siderophore receptor proteins, including FpvA (*P. aeruginosa* pyoverdine receptor), PupA (*Pseudomonas putida* WCS358 pseudobactin 358 receptor) and FhuE (*E. coli* coprogen, rhodotorulic acid and ferrioxamine E receptor).

1.4.3. The Role of Iron in Pyochelin Biosynthesis

Production of both pyochelin and the ferripyochelin receptor is strongly regulated by PchR (Heinrichs and Poole, 1993). *PchR* has strong homology to the AraC family of transcriptional activators found in bacteria including *E. coli*, *Salmonella typhimurium*, *Citrobacter freundii* and *Erwinia chrysanthemi*. There is a DNA sequence upstream of *pchR* that has homology with the *E. coli* Fur (ferric uptake regulator - section 1.5.5.) recognition sequence suggesting that PchR production is iron-regulated i.e. iron is responsible for mediating the level of expression of an activator protein (PchR) rather than directly affecting the structural genes for siderophore and receptor. PchR is also required for pyochelin induction of the ferripyochelin receptor. This suggests that PchR may undergo posttranscriptional regulation by pyochelin in activating receptor synthesis and may also upregulate pyochelin synthesis in response to the siderophore. The combination of PchR and Fur imply that pyochelin and FptA synthesis are subjected to both positive and negative siderophore specific regulation (Heinrichs and Poole, 1993).

Other work regarding siderophore induction of OMP expression was performed by Gensberg *et al.* (1992). It was noted that addition of purified siderophore to the growth medium of siderophore-deficient strains of *P. aeruginosa* induced expression of specific IROMPs with a subsequent increase in the rate of iron transport (Gensberg *et al.*, 1992). For example, addition of pyochelin increased ferripyochelin transport but not ferripyoverdine transport with a corresponding increase in expression of the 75 kDa IROMP. Addition of pyoverdine induced expression of an 85 kDa IROMP with an increase in ferripyoverdine transport. Evidently, in addition to iron-limitation, *P. aeruginosa* can also respond to the presence of siderophores. This may explain why *P. aeruginosa* retains two siderophore systems even though the iron binding ability of pyoverdine is much greater than pyochelin. It was postulated that both siderophores are produced initially in low quantities, followed by further de-repression of the system most suited to that particular environment (Gensberg *et al.*, 1992). The ability to use two systems may also protect against the possibility of mutation removing one of the iron uptake systems. A similar induction system was observed by Poole *et al,* (1990) when growth of *P. aeruginosa* in the presence of the *E. coli* siderophore enterobactin resulted in the expression of an iron-uptake system responsible for ferrienterobactin transport (section 1.7.). In *P. putida* WCS358, the PupB receptor which is associated with ferripseudobactin BN8 and ferripseudobactin BN7 transport appears to be involved with initiation of a signal transduction pathway leading to its own synthesis (Koster *et al.*, 1994) *via* a positive regulator element PfrA. This is similar to some periplasmic-binding-protein-dependent transport systems like the phosphate specific Pst system, which in addition

to their transport function, have a role in signal transduction (Cox *et al.*, 1988). PupB is the first example of an IROMP playing this role.

1.4.4. Pyoverdine

P. aeruginosa also produces one of several pyoverdine siderophores related to the pseudobactins produced by other fluorescent pseudomonads. Pyoverdines are highly water soluble fluorescent compounds giving rise to the characteristic green-yellow culture supernatants observed when *P. aeruginosa* is grown under iron-limiting conditions.

All pyoverdines share a dihydroxyquinolone chromophore and two N-hydroxyornithines attached to a varying amino acid backbone (Albrecht-Gary *et al.*, 1994). Pyoverdine PaA of *P. aeruginosa* is a 6,7-dihydoxyquinoline-containing fluorescent chromophore bound to the N-terminus of an octapeptide (D-Ser-L-Arg-D-Ser-L-N5-OH-Orn-L-Thr-L-Thr-L-Lys-L-N5-OH-Orn) (Jego *et al.*, 1993). Transport of the different ferripyoverdine and ferripseudobactin complexes is *via* corresponding IROMPs. The varying specificities of IROMPs towards the various pyoverdines has been attributed to subtle differences in the amino acid backbone of the siderophores (Poole *et al.*, 1991).

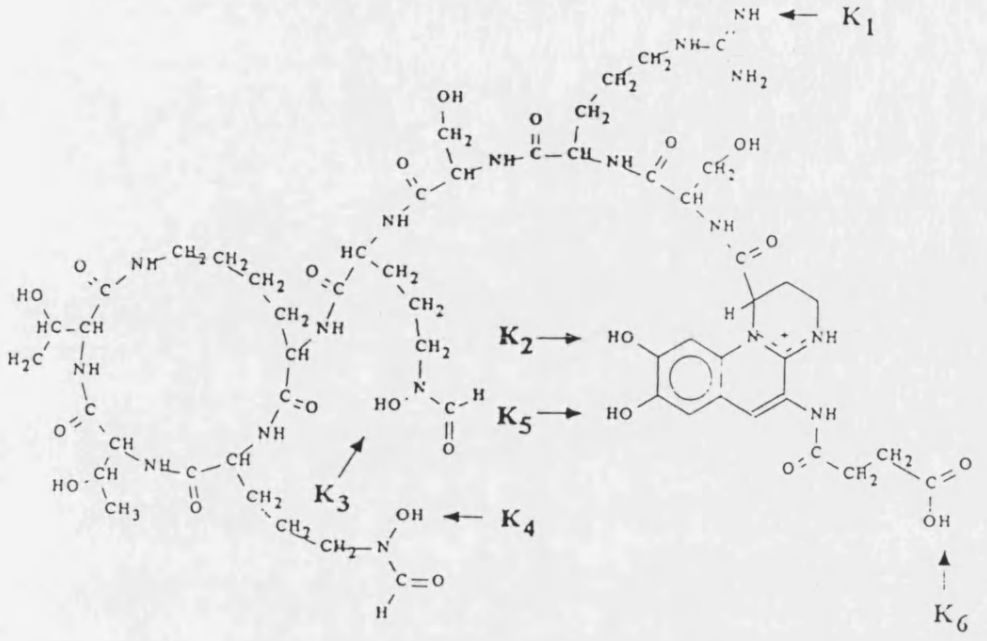


Figure 1.3. The structure of the fully protonated form of pyoverdine PaA (Albrecht-Gary *et al.*, 1994).

Poole *et al.*, (1991) reported a 90 kDa IROMP associated with ferripyoverdine uptake. Loss of this IROMP was concomitant with a loss of both ferripyoverdine transport and pyoverdine production. However, a mutant deficient in the production of this 90 kDa OMP always transported very low levels of ferripyoverdine. Similarly, the 90 kDa deficient strain also demonstrated pyoverdine-dependent growth on EDDHA-supplemented media suggesting a second uptake system may exist. In addition, similar phenomena were noted in an 85 kDa OMP mutant deficient in the transport of ferripyoverdine which showed a 5% residual ferripyoverdine uptake (Smith *et al.*, 1992). The mutant deficient in the 85 kDa protein appeared to compensate by over-production of the 75 kDa protein associated with ferripyochelin uptake.

The ferripyoverdine receptor gene, *fpvA*, was cloned by Poole *et al.*, (1993b). The deduced 90 kDa polypeptide has homology to regions shown to be conserved in TonB-dependent receptor proteins (section 1.6.1.). There is particularly strong homology with the PupA protein of *P. putida* WCS358 which is the receptor for ferripseudobactin WCS358. FpvA also shares a large degree of homology with FhuE, the *E. coli* receptor for ferric coprogen, ferric rhodotorulic acid and ferrioxamine B which is a little more surprising considering the pyoverdines and pseudobactins are structurally different to these molecules. However, they do all share hydroxyornithine residues (Winkelmann, 1986).

1.4.5. The Role of Iron in Pyoverdine Biosynthesis.

A large number of genes are involved in pyoverdine biosynthesis and it is proposed that they have evolved from a common ancestral origin (Rombel and Lamont, 1992). The most studied species are plant-growth-enhancing *P. putida*, plant pathogen *Pseudomonas syringae*, *Pseudomonas* sp M114 and *P. aeruginosa.*

All genes associated with pyoverdine production in *P. aeruginosa* have been isolated in the 47' position on the chromosomal map, termed the *pvd* region (Tsuda *et al.*, 1995). Pyoverdine synthesis appears to be directed from this region and expression of some of the *pvd* genes is regulated at the level of transcription (Miyazaki *et* al., 1995).

PvdS has been identified as a positive regulator of several genes in the *pvd* region and was isolated by Cunliffe *et al.*, (1995) and Miyazaki *et al.*, (1995) independently. *PvdS* is required for expression of at least two promoters of pyoverdine synthesis and is suspected of acting at many other promoters of pyoverdine production that have yet to be characterised. *PvdS* mutants are unable to synthesise pyoverdine due to the lack of transcription from the *pvd* promoters (Cunliffe *et al.*, 1995). *PvdS* shares significant homology to RNA polymerase sigma factors produced by *E. coli* which direct the synthesis of extracellular products. There is strong homology towards *E. coli fecI* which is a positive regulator of the *fec* (ferric citrate transport) operon and *P. putida* PupI, a positive regulator of the *pupB* gene (van Hove *et al.*, 1990; Koster *et* al.,1994). FecI and PupI are modulated by FecR and PupR, respectively, although it is not known whether there is a similar modulator for *P. aeruginosa.* However, the promoter region of *pvdS* is similar to those of the *pupI* and *fecI* operons and has an area with strong homology to the Fur box in *E. coli.* Consequently, transcription of the *pvdS* promoter is repressed by the presence of iron so that in the absence of iron, Fur is unable to bind *pvdS* leading to pyoverdine biosynthesis (Cunliffe *et al.*, 1995).

Similarities to other species include homology between *pvdS* and *pbrA* from *P. fluorescens* M114 (Sexton *et al.*, 1995). With *P. putida*, at least 15 genes in five gene clusters are required for pseudobactin synthesis (Marugg *et* al., 1985) and similar numbers have been identified in *Pseudomonas sp.* B10 (Moores *et al.*, 1984) and *P. syringae* (Loper *et al.*, 1984). It is thought that *P. aeruginosa* will have a similarly complex genetic composition.

PvdA is another gene involved in pyoverdine biosynthesis and encodes the enzyme L-ornithine N5 oxygenase. This enzyme catalyses the hydroxylation of L-ornithine to N5-hydroxylornithine, a component of the peptide moiety (Visca *et al.*, 1994). Finally, PvdD is proposed to be involved in the formation of the octapeptide by a non-ribosomal method (Merriman *et al.*, 1995).

1.4.6. Pyoverdine Secretion

Less is known regarding the secretion of pyoverdine, although Poole *et al.*, (1993a) proposed the involvement of an operon encoding three proteins OprK, MexA and MexB. OprK is a 50 kDa OMP which has homology with several export proteins, MexB is a 108 kDa cytoplasmic membrane protein which acts as an efflux pump and MexA is a 40 kDa periplasmic protein thought to link OprK and MexB. A similar system comprising the 50 kDa OprM, the 46 kDa MexC and the 100 kDa MexD (Li *et al.*, 1994) has been suggested to play a role in the secretion of pyochelin (Hamzehpour *et al.*, 1995). Both systems were first identified in association with multidrug efflux which enable bacteria to avoid the harmful effects of antimicrobial agents. Antimicrobials thought to be excreted by these systems include the quinolones, tetracycline and chloramphenicol (Poole *et al.*, 1993a; Hamzehpour *et al.*, 1995).

1.5. Emerging Themes in Gene Regulation in *Pseudomonas aeruginosa*

In addition to the siderophores produced in response to iron deprivation, *P. aeruginosa* can also produce several virulence factors responsible for damage to the

host. This section identifies some of the emerging trends in the genetic regulation of this organism, with particular respect to the role of iron.

1.5.1. The Role of Iron in Exotoxin A Biosynthesis

Exotoxin A (ETA) is one of the major virulence factors of *P. aeruginosa.* This enzyme, which is toxic to animals at the microgram or submicrogram level (reviewed by Vasil *et al.*, 1990), is an ADP-ribosyltransferase toxin that inhibits protein synthesis in eukaryotic cells in a similar manner to diphtheria toxin. It is a classical A-B toxin, with a domain designated B which is required for binding on the eukaryotic cell surface and a domain designated A which is responsible for the toxic activity. The production of ETA is highly regulated (Storey *et al.*, 1991) with maximal expression occurring under iron-limiting conditions such as those in the human host. Regulation of the structural gene, *toxA,* is influenced by the *regA* gene and its products. A second gene, termed *regB* is required for optimal ETA production (Wick *et* al., 1990). There is also evidence that *toxA* activity is enhanced by LasR, the transcriptional regulator originally associated with elastase production (Gambello and Iglewski, 1991). The ability of RegA to bind RNA polymerase suggests that it may be an alternative sigma factor (Walker *et al.*, 1994) (section 1.5.4.).

1.5.2. Exoenzyme S

Exoenzyme S (ExoS), like ETA, is an ADP-ribosyltransferase enzyme although their synthesis and excretion are independent. ExoS has been isolated in approximately 90% of clinical isolates suggesting a clear role in pathogenesis (Goranson and Frank, 1996). ExoS production is dependent upon growth conditions, suggesting that it is

produced in response to environmental stimuli such as stress and temperature (Frank and Iglewski, 1991). However, the availability of iron has little effect. Interestingly, yields are reduced by ciprofloxacin, tobramycin and ceftazidime at sub-MIC concentrations (Grimwood *et al.*, 1989).

1.5.3. Quorum Sensing and the Role of Elastase in Iron Acquisition

Elastase is an important virulence factor that contributes to the pathogenicity of *P. aeruginosa*. It is a metalloprotease with a broad substrate-specificity attacking host molecules including elastin, collagen, immunoglobulins and some components of the complement cascade. Elastase, therefore, releases iron to the bacterium and inactivates the host immune response (Iglewski *et al.*, 1990).

In addition, elastase also cleaves transferrin and lactoferrin which are normally found in the lung and contribute to the host defence mechanisms. Iron released as a result of transferrin and lactoferrin cleavage is scavenged by the siderophore pyoverdine. This also generates new iron chelates which, in contrast to transferrin-bound iron, are able to catalyse formation of highly cytotoxic radicals from neutrophil-derived superoxide and hydrogen peroxide *via* the Haber-Weiss reaction (Britigan *et al.*, 1993). This cleavage can work in tandem with neutrophil-derived proteases contributing to *P. aeruginosa*-associated lung injury by digesting the extracellular matrix (Fick and Hata, 1989). In addition, pyocyanin, a blue-coloured secretory product of *P. aeruginosa*, previously associated with reducing ciliary activity (Jakowski *et al.*, 1991) has the ability to undergo redox cycling under aerobic conditions resulting in the formation of superoxide and hydrogen peroxide. Iron

bound to the siderophore pyochelin can generate free radicals as a result of Haber-Weiss chemistry and the combination of pyocyanin and ferripyochelin work synergistically in mediating epithelial cell damage (Britigan *et al.*, 1992).

Optimum elastase production occurs at late-log and stationary growth phases. Although the exact environmental signals are unclear, iron-deplete conditions appear to have a weak positive effect on elastase production. However, elastase and several other exoproducts of *P. aeruginosa* are not produced until the cell density is relatively high and is understood to operate *via* an autoinducer-responsive transcriptional regulatory system termed "Quorum Sensing" (Latifi *et al.*, 1995). The bacteria produce a diffusible compound, the autoinducer, which accumulates in the growth medium. The bacterium is freely permeable to the autoinducer so that at low levels the compound diffuses out of the cell and therefore has no effect. However, at high cell densities, the autoinducer accumulates above critical levels required for expression of the respective genes (reviewed by Fuqua *et al.*, 1994). This type of system was first isolated in the marine bacterium *Vibrio fischeri*, which utilises the LuxI-LuxR autoinduction system for expression of luminescence genes. The *Vibrio* autoinducer is termed VAI (Nealson *et* al., 1970). This self-stimulating regulatory mechanism has the potential to generate a strong response to appropriate stimuli by co-ordinating the response from an entire population of cells *via* autoinducer production.

P. aeruginosa elastase production is subject to quorum sensing regulation and several systems have now been described. One system relies upon the interaction of

LasR, a cell density-dependent transcriptional activator of several genes required for virulence, and the *Pseudomonas* autoinducer (PAI), an N-acyl-L-homoserine lactone, which is synthesised under the direction of LasI, the cell density-dependent signal generator. LasR and LasI show distinct homology with LuxR and LuxI of *V. fischeri* (Gambello and Iglewski, 1991; Passador *et al.*, 1993). LasR is a global regulator that activates transcription of several genes including *lasB* which codes for elastase production, *lasA* which codes for LasA protease production and *aprA* which codes for alkaline protease. LasR also enhances transcription of the exotoxin A gene, *toxA* (Gambello *et al.*, 1993).

Briefly, PAI interacts with LasR to form a complex which interacts positively with promoters of *lasI*. This positive regulation of *lasI* by the PAI-LasR complex results in enhanced PAI production until a saturating concentration is reached i.e. a positive feedback loop (Seed *et al.*, 1995). *LasI* requires significantly lower concentrations of PAI for expression than are required by *lasB*, therefore with low levels of PAI, the PAI-LasR complex interacts with the higher affinity *lasI*, generating further amounts of LasI. Larger amounts of LasI result in additional amounts of PAI which would accumulate to such a level where enough activated LasR would be present to associate with the lower affinity elements such as the *lasB* elements. This system, whereby *lasI* and *lasB* are activated by different levels of PAI suggest a two tier autoinduction hierachy (Seed *et al.*, 1995).

The first N-acyl-L-homoserine lactone to be identified in culture supernatants of *P. aeruginosa* was N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (Bainton *et al.*,

1992). Jones *et al.* (1993) demonstrated that OHHL was able to restore partial elastase synthesis in pleiotropic mutants unable to synthesise a number of virulence factors. It was later discovered that LasI also directed the synthesis of a second N-acyl-L-homoserine lactone, N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) (Pearson *et* al., 1994) although this compound was unable to restore elastase synthesis in pleiotropic mutants (Latifi *et al.*, 1995). However, a genetic locus was identified that could complement the pleiotropic mutant and restore elastase production. The locus encodes LuxR and LuxI homologues termed VsmR (virulence determinants and secondary metabolites) and VsmI, which were distinct from LasR and LasI (Winson *et al.*, 1995). These genes have also been termed RhlR and RhlI (Latifi *et al*, 1995). VsmI directs the synthesis of two N-acyl-L-homoserine lactone molecules which have have been identified as N-butanoyl-L-homoserine lactone (BHL) and N-hexanoyl-L-homoserine lactone (HHL) which are found in the ratios of 15:1 in *P. aeruginosa* culture supernatants (Winson *et al.*, 1995). In addition, BHL is more potent at restoring elastase production suggesting that this is the natural ligand, although it is possible that HHL regulates other systems that are as yet unexamined.

Multiple autoinducer systems have been reported in *Vibrio harveyi* (Bassler *et al.*, 1994) and *Erwinia carotovora* (Swift *et al.*, 1993). However, *P. aeruginosa* is the first organism in which two LuxR homologues (LasR and VsmR/RhlR) and two LuxI homologues (LasI and VsmI/RhlI) have been described along with their respective autoinducers. Consequently, the roles played by each homologue and the proposed interactions between them suggests that quorum sensing in *P. aeruginosa*

is emerging to form a complex system regulating virulence factor and metabolite production in response to cell population density (Latifi *et al.*, 1995).

The success of *P. aeruginosa* as a pathogen appears to result in part from its ability to regulate virulence factor production in response to environmental signals. Quorum sensing systems would appear to allow it to launch an aggressive attack on the host in areas of high cell density such as alveolar abscesses (Brint and Ohman, 1995).

1.5.4. The Role of Alternative Sigma Factors

The involvement of alternative sigma factors in the genetic regulation of *P. aeruginosa* is becoming increasingly evident. One particularly interesting factor thought to function as an alternative sigma factor is PvdS which is iron regulated and directs several elements of pyoverdine biosynthesis (section 1.4.6; Cunliffe *et al.*, 1995). In addition, conversion of *P. aeruginosa* to the exopolysaccharide alginate-overproducing mucoid form is also thought to depend upon the alternative sigma AlgU (Deretic *et al.*, 1994).

Alginate overproducing forms are rarely found in environmental strains whereas strains isolated from the CF lung are frequently mucoid. It is an O-acetylated linear polymer of D-mannuronate and L-guluronate residues (Evans and Linker, 1973), which is thought to suppress leukocyte function and nonopsonic phagocytosis, promote adhesion and scavenge reactive oxygen intermediates (Deretic *et al.*, 1994).

Alginate is produced from a complex pathway involving several biosynthetic enzymes. *AlgD* encoding phosphomannose dehydrogenase, undergoes strong transcriptional up-regulation in mucoid cells. All genes in the alginate biosynthesis cluster are transcribed in the same direction as *algD* and and all appear to be regulated by the *algD* promoter (Chitnis and Ohman, 1993). Factors affecting *algD* regulation include dehydration, osmolarity, nutrient deprivation, oxygen availability and slow growth rate and are mediated *via* the activities of AlgR and AlgU. Ultimately, control of *algD* expression is via the *algU*, *mucA*, *mucB* gene cluster. *AlgU* (also termed *algT*-Martin *et al.*, 1993a) is most likely an alternative sigma factor required for initiation of *algD* transcription as the gene product has 77% homology to the sigma factor σ^E of *E. coli* (DeVries and Ohman, 1994).

MucA and MucB normally repress *algU* expression although in a significant number of CF isolates, *mucA* is inactivated by frameshift and nonsense mutations (Martin *et al.*, 1993b). Mutations in *mucA* or *mucB*, result in derepression of alginate sysnthesis leading to a conversion to mucoidy. MucA and MucB have been proposed to act in a manner similar to anti-σ factors which prevent the σ factors interacting with the core RNA polymerase (Deretic *et al.*, 1995).

1.5.5. The Role of Fur in Iron Regulation

As noted in section 1.4., *P. aeruginosa* responds to conditions of iron deprivation by the derepression of high affinity iron uptake systems based on the siderophores pyoverdine and pyochelin. Production of pyochelin and the respective ferripyochelin receptor are tightly iron regulated and appear to be under the control of the

transcriptional activator PchR (section 1.4.3.). Similarly, PvdS is involved in the transcription of at least two pyoverdine biosynthesis genes and its production depends on iron deprivation (section 1.4.6.). Finally, virulence factors such as exotoxin A have also been demonstrated to be tightly iron regulated *via* the *regAB* operon (section 1.5.1.) Global regulation of these systems has been attributed to the 15.2 kDa Fur (ferric uptake regulator) protein (Prince *et al.*, 1993). It is proposed that Fur does not interact directly with the biosynthetic genes but instead regulates the expression of the positive regulatory factors associated with siderophore and exotoxin biosynthesis i.e. PchR, PvdS and RegAB. The *P. aeruginosa fur* gene was cloned and sequenced by Prince *et al.* (1993) and its effects on iron-regulated promotors characterised by Ochsner *et al.* (1995).

The role of iron in genetic regulation is best understood in *E. coli* where the Fur protein is one of the major regulatory components. The presence of the *fur* gene was first described in mutants of *Salmonella typhimurium* (Ernst *et al.*, 1978) and later in *E. coli* by Hantke (1981). *E. coli fur* was sequenced by Schaffer *et al.*, (1985) and the gene product, Fur, identified as the component responsible for the repressor effect (Wee *et al.*, 1988).

E. coli Fur is a 17 kDa protein (Schaffer *et al.*, 1985) responsible for the global regulation of many siderophore-dependent iron-uptake systems and virulence factor production (Venturi *et al.*, 1995). *In vitro* experiments demonstrated that the absolute exclusion of oxygen was required for the operation of Fur suggesting that the biologically active metal was Fe(II) and not Fe(III). Fur is, therefore, a metal-

regulated protein rather than a metalloprotein i.e. Fur is normally isolated in the metal-free state and will accept cations as activators. In contrast, metalloproteins are normally isolated containing a stoichiometric amount of a specific metal ion. An important factor in the operation of Fur and its co-repressor Fe (II), is that the interior of the cell must be highly reducing in order to reduce Fe (III) to Fe (II) (Bagg and Neilands 1987a).

Fe (II) acts as a co-repressor. The Fur-Fe(II) complex binds to a consensus sequence, GATAATGATAATCATTATC, termed the "Fur Box" located in the promoter regions of iron-regulated genes. In iron replete conditions, the Fur-Fe(II) complex inhibits transcription from these promoters, hence preventing expression of iron-regulated genes. In iron depleted conditions, Fur and Fe (II) dissociate leading to derepression of Fur-dependent promoters and an increase in expression of iron-regulated genes (Bagg and Neilands 1987a). Strains with *fur* mutations display constant derepression of iron-regulated systems at concentrations of iron that normally repress their production (Calderwood and Mikalanos, 1987). Interestingly, it has never been possible to isolate *fur*-null mutants in *P. aeruginosa* suggesting that loss of this global regulator may be lethal (Prince *et al.*, 1993; Ochsner *et al.*, 1995)

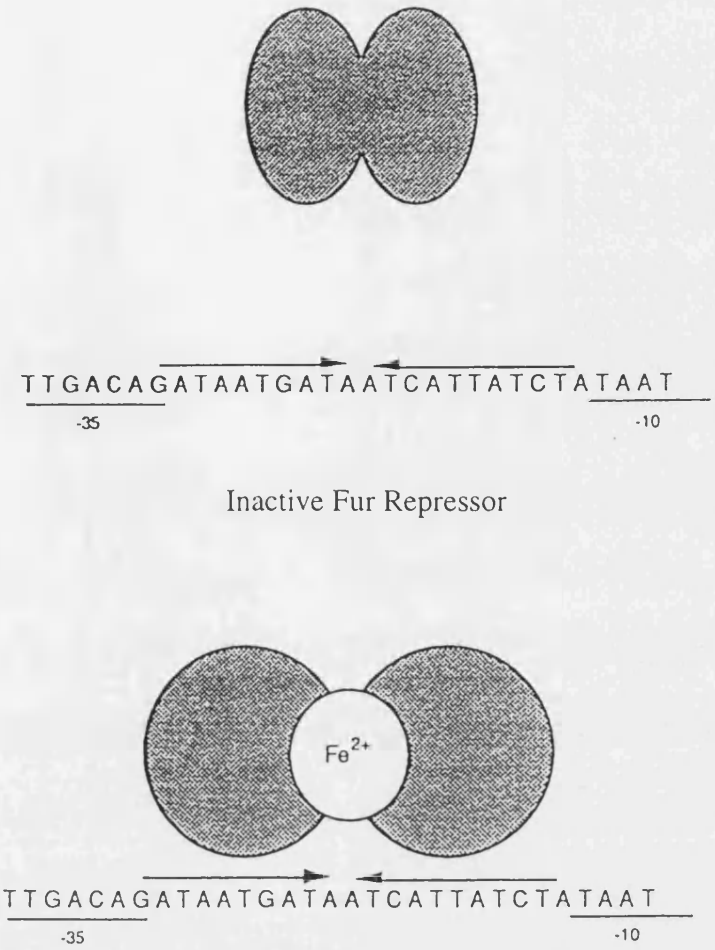


Figure 1.4. A diagrammatic representation of the Fur repressor system (Litwin and Calderwood, 1993).

Fur homologues have now been isolated in several other species, including *Salmonella typhimurium* (Foster and Hall, 1992), *Yersinia pestis* (Staggs and Perry, 1991), *Vibrio cholerae* (Litwin *et al.*, 1992), *Vibrio vulnificas* (Litwin and Calderwood, 1993) and *P. putida* (Venturi *et al.*, 1995). There is considerable homology between the Fur homologues of different species with the exception of *P. aeruginosa*. *P. aeruginosa* Fur is only 53% identical to *E. coli* Fur and 49% identical

to *V. cholerae* and *Yersinia pestis* Fur. Most of the differences occur in the carboxy terminus of the protein which, in *E. coli*, is the metal binding domain (Prince *et al.*, 1993).

1.6. Models of Iron Transport Across the Gram-Negative Cell Wall

Following the release of iron-scavenging siderophores into the environment, the iron-siderophore complexes have to be internalised and the iron liberated to the interior of the bacterium. In Gram-negative bacteria, this requires transport across the structurally complex cell wall, the first barrier being the relatively impermeable outer membrane.

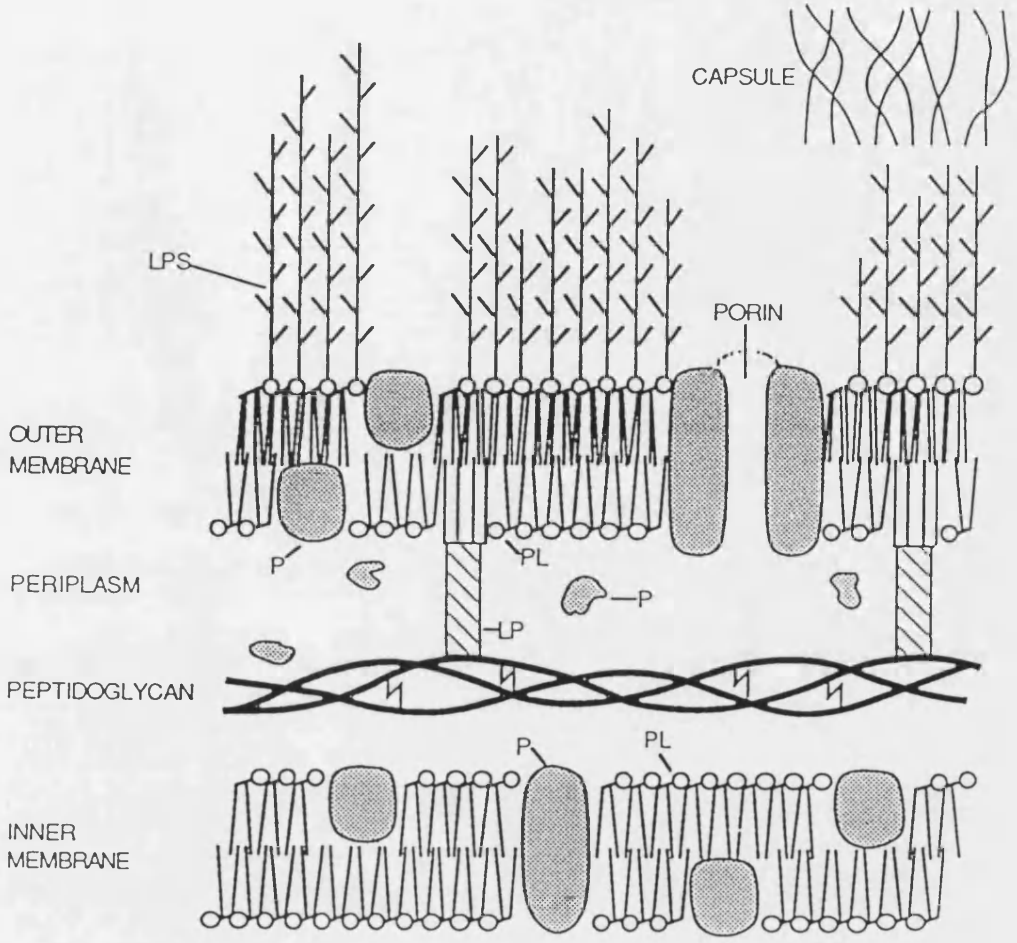


Figure 1.5. Schematic representation of the Gram-negative cell wall. LPS = lipopolysaccharide; PL = phospholipid; P = protein; LP = lipoprotein (Adapted from Alberts *et al.*, 1994).

1.6.1. Iron Transport Across the Outer Membrane

The outer membrane of Gram-negative bacteria is a non-classical lipid bilayer that allows passage of small hydrophilic molecules into the periplasm *via* trimeric porin proteins spanning this bilayer, (Nikaido, 1982). The outer leaflet comprises LPS and the inner leaflet is largely phospholipid (Postle, 1990a). The periplasm is an aqueous

environment containing several detoxifying enzymes, scavenging enzymes and binding proteins responsible for facilitating active transport. Peptidoglycan gives the cell rigidity and protects the bacterium from osmotic stress (Hancock and Bell, 1988). The cytoplasmic membrane is the site of several proteins responsible for the generation and maintenance of electrochemical potential as well as those proteins which use the electrochemical potential for active transport of nutrients into the cell (Postle, 1990a).

The Gram-negative envelope confers a large degree of protection from a wide range of antibiotic compounds, digestive enzymes and various components of the host immune response. However, the success of OM impermeability provides an obstacle for transport of nutrients into the cell. Ferri-siderophore complexes and vitamin B_{12} cannot be transported *via* porin pathways and rely on active transport processes involving the TonB protein.

1.6.1.1. The Role of TonB

TonB couples energy from the cytoplasmic membrane and facilitates active transport of iron-siderophore complexes and vitamin B_{12} across the outer membrane. This enables Gram-negative bacteria to benefit from the impermeability of the OM yet still allowing the passage of certain essential nutrients into the cell (Postle, 1990a).

The majority of work on TonB has been performed in *E. coli*. *E coli* TonB is a 26 kDa protein (Postle and Good, 1983) rich in proline residues at the amino terminus. *TonB* mutants are effectively iron-limited and therefore hyper-express their high

affinity iron-uptake systems. *TonB* expression is regulated by iron and Fur (Postle, 1990b) and fails to be expressed under anaerobic conditions. This is probably a result of the free solubility of ferrous iron in anaerobic conditions which is transported in a TonB-independent manner (Hantke, 1987). Fur binds *in vivo* to the "Fur Box" located between the -35 and -10 regions of the TonB promoter to reduce TonB transcription in iron-replete media (Postle, 1993).

Predicted structure analysis suggests that TonB has two hydrophobic regions, one at each terminus, whilst the central region is hydrophilic (Poste and Good, 1983). The hydrophobic amino terminus is anchored in the cytoplasmic membrane and the majority of the hydrophilic region extends into the periplasm (Postle and Skare 1988).

Briefly, a TonB-dependent transport system comprises an outer membrane receptor, a periplasmically located binding protein and a complex of cytoplasmic membrane proteins for active transport of ligands across the cytoplasmic membrane. The outer membrane receptors are essentially gated porins, closed at the cell surface by their ligand binding peptides (Klebba *et al.*, 1993) and that opening and closing of TonB-dependent channels is an energy-dependent phenomenon (Woolridge *et al.*, 1992). All TonB-dependent outer membrane receptors show distinct regions of homology where the TonB protein is proposed to make contact termed the "TonB boxes" (Kadner, 1990). However, TonB itself does not participate in transport across the cytoplasmic membrane (Postle, 1993).

One popular hypothesis of TonB function is that in the energised state, TonB spans the periplasm, touching the underside of outer membrane receptors at the periplasmic face of the OM. Energised TonB induces a change in conformation of the receptors causing them to release their substrate to periplasmic binding proteins (Rutz *et al.*, 1992). Induction of receptor-release consumes energy so that the TonB protein switches back to the unenergised conformation and has to be re-energised by cytoplasmic membrane potential. This strengthens the suggestion that there is a physical interaction between the TonB protein and the relevant receptors (Braun *et al.*, 1991). In addition, competition exists between the different TonB-dependent receptors for the limited amounts of functional TonB. Kadner and Heller (1995) noted that mutual inhibition of vitamin B_{12} and ferrisiderophore uptake is overcome by increasing the availability of functional TonB.

1.6.1.2. TonB-Associated Proteins

Other proteins associated with TonB activity are ExbB and ExbD encoded by the *exb* operon. ExbB is a 26 kDa cytoplasmic membrane protein with its amino terminus protruding into the periplasm (Eick-Helmerich and Braun, 1989). The majority of ExbB is found in the cytoplasm and appears to stabilise the TonB protein. Postle and Skare (1988) demonstrated that plasmid-encoded TonB had a functional half-life of 10 min which was reduced to 2 min in a strain with an *exbB* mutation. Overexpression of TonB can compensate for an *exbB* mutation whereas overexpression of ExbB cannot compensate for a TonB mutation (Fischer *et al.*, 1989). It appears that ExbB offers proteolytic protection, probably by its ability to convert TonB to a protease-resistant conformation (Skare *et al.*, 1993). However,

with *exbB* mutations, there still remains approximately 10% residual TonB-dependent activity which is most likely a result of crosstalk with the Tol system which is involved with the uptake of biopolymers (Braun *et al.*, 1991). The *exb* locus has two reading frames whilst the *tol* locus has four, designated *tolQRAB*. ExbB has 26% identity and 79% similarity with the TolQ sequence and ExbB has 25% identity and 70% similarity to TolR. The similarities of ExBD to TolQR proteins suggest a common ancestry. Whilst mutations in *exbBD* did not completely abolish TonB activity, activity was lost upon a double mutation of both *exbBD* and *tolQ* (Braun and Herrmann, 1993). It is thought that there is a mechanical interaction between TonB and ExbB/TolQ proteins (Eick-Helmerich and Braun, 1989).

Less is known about ExbD. It is found in both the cytoplasmic membrane and periplasm, with the majority, like TonB, being in the periplasm (Postle, 1993). In addition, ExbB binds ExbD and prevents degradation by proteases (Fischer *et al.*, 1989). Ahmer *et al.* (1995) noted that *exbB* and *exdD* were so close that they appear to be co-ordinately expressed as a single operon and it is most like that they are co-transcribed from the *exbB* promoter. The phenotypes of strains lacking either intact ExbB or ExbD proteins are indistinguishable, suggesting that the roles they play in TonB stabilisation are interchangeable.

Ligand release is thought to be precipitated by detachment of TonB from the outer membrane receptor due to a conformational change. ExbB and ExbD are thought to recycle TonB back to an active conformation. The ligand is then free to interact with the periplasmic binding proteins followed by transport across the cytoplasmic

membrane. The TonB system includes all of the ferric-siderophore receptor proteins known for *E. coli* including FepA (enterobactin), IutA (aerobactin), FecA (ferric citrate), FhuA (ferrichrome) and FhuE (rhodoturulic acid) (reviewed by Guerinot, 1994).

1.6.1.3. TonB Homologous Systems in Other Gram-Negative Bacteria

A TonB homologue has recently been identified in *P. aeruginosa* (Poole *et al.*, 1996). *P. aeruginosa* TonB appeared novel compared to previously described TonB proteins since it lacked an N-terminal membrane anchor sequence. However, it does appear to have an N-terminal extension located in the cytoplasm although the function of this is unknown. It was noted that *P. aeruginosa* TonB shared 31% and 21% identity with the TonB proteins of *E. coli* and *P. putida*, respectively, and was able to complement *tonB* mutaions in both of these organisms (Poole *et al.*, 1996).

TonB homologues have been identified in several other Gram-negative organisms including *Salmonella typhimurium* (Hannavey *et al.*, 1990), *Klebsiella pnuemoniae* (Bruske *et al.*, 1993), *Enterobacter aerogenes* (Bruske and Heller, 1993), *Serratia marsescens* (Gaisser and Braun, 1991), *Yersinia enterocolitica* (Koebnik *et al.*, 1993), and as noted previously, *Pseudomonas putida* (Bitter *et al.*, 1993). In *Haemophilus influenzae*, a 28 kDa TonB homologue was found to be required for utilisation of haem (Jarosik *et al.*, 1994). All have similar sequences, with substantial runs of identical residues. Heterologous *tonB* genes from many of the enteric bacteria function to some extent in *E. coli* (Postle, 1993) although *P. putida* and *E. coli* TonB proteins are not functionally interchangeable (Bitter *et al.*, 1993).

Vibrio cholerae does not appear to have a TonB homologue although the outer membrane haem transport protein, HutA, was found to have a region displaying strong homology with the TonB box from TonB-dependent receptors. It is thought however, that a TonB-like protein may exist, although this has yet to be isolated (Henderson and Payne, 1994).

1.6.2. Transport Across the Cytoplasmic Membrane

The final barrier for the ferri-siderophore complex is passage across the cytoplasmic membrane. Most high-affinity iron-uptake systems in Gram-negative bacteria are thought to use periplasmic-binding-protein-dependent transport and one of the most widely studied systems is enterobactin-mediated iron transport in *E. coli*. The ferrienterobactin complex interacts with FepA which is located in the outer membrane and as result of TonB activity is transfered to the periplasm. The ferrienterobactin complex then becomes bound to the periplasmic binding protein FepB which in turn presents the complex to the FepC, D and G proteins. These proteins use energy derived from the hydrolysis of ATP to promote the passage of the iron-siderophore complex into the cytoplasm (Stephens *et al.*, 1995). With the exceptions of siderophore complexes and vitamin B_{12}, which have specific outer membrane receptors, these periplasmic systems serve as the initial receptor for active transport of substrates into the bacteria. The overall system is similar to that used in the transport of peptides, amino acids and sugars (Nikaido and Saier, 1992). The usual arrangement of proteins comprises a periplasmic binding protein, one or two very hydrophobic proteins located in the cytoplasmic membrane and a hydrophilic membrane associated protein with recognition sequences for nucleotide binding such

as ATP (Angerer *et al.*, 1990). Transport across the cytoplasmic membrane is therefore considered to occur independently of outer membrane transport (Schoffler and Braun, 1989) (for further details see chapter 7).

1.6.3. Iron Release from Siderophores

The final stage of iron transport is the release of iron from the siderophore. Again the most studied system is enterobactin-mediated iron transport in *E. coli*, with release thought to take place either at the inner face of the cytoplasmic membrane or within the cytoplasm itself. However, the actual mechanism of iron release from siderophores is relatively poorly understood.

1.6.3.1. Cytoplasmic Esterase-Mediated Iron Release

It was thought that *E. coli* was able to mediate iron release through the activity of a cytoplasmic esterase. Enterobactin which is a trimer of dihydroxybenzoylserine (O'Brien and Gibson, 1970), is hydrolysed by this enzyme with a subsequent release of iron (O'Brien *et al.*, 1970). However, the observation that a hydrolysis-resistant, synthetic analogue of enterobactin, N,N',N''-tri(1,3,5-tris)-2,3-dihydroxybenzoylaminomethylbenzene could still release iron to *E. coli*, suggested that other processes could be in operation (Heidinger *et al.*, 1983).

1.6.3.2. Reductase-Mediated Iron Release

It is now well documented that reduction of siderophore-bound iron from Fe(III) to Fe(II) results in release of the iron from siderophores due to the low affinity of siderophores towards Fe(II). Ferrisiderophore-reductase activity has been reported in

a variety of organisms including *E. coli* (Fischer *et al.*, 1990), *Bacillus subtilis* (Gaines *et al.*, 1981) and *P. aeruginosa* (Halle and Meyer, 1992a ; Cox, 1980). In describing the reductase associated with ferripyoverdine activity, Halle and Meyer (1992a) noted that the reductase is a soluble enzyme located in the cytoplasm. Immunological screening also identified this enzyme in 18 *Pseudomonas* strains including *P. stutzeri*, a non-fluorescent species, suggesting that it is ubiquitous amongst *Pseudomonads*. Later work by Halle and Meyer (1992b) found that the proposed ferripyoverdine reductase reduced numerous ferri-complexes suggesting that the *P. aeruginosa* reductase is a non-specific enzyme which may be involved in several reductase pathways (see chapter 6).

1.7. Exogenous Siderophore-mediated Iron Transport by *Pseudomonas aeruginosa*

In additon to the endogenous siderophores pyochelin and pyoverdine, several exogenous compounds have siderophore activity in *P. aeruginosa*. These include siderophores produced by other species of bacteria and fungi such as ferrioxamine B, the siderophore of *Streptomyces* spp. (Cornelis *et al.*, 1987). Other exogenous compounds proposed to have roles in mediating iron uptake include citrate and salicylate. A 43 kDa OMP has been postulated to be the ferric-citrate receptor (Harding and Royt, 1990) although a receptor has yet to be assigned to the ferrisalicylate complex. However, since salicylate is both a precursor to, and part of the structure of pyochelin, interaction may be *via* the 14 and 75 kDa IROMPS (Meyer, 1992). In addition to the proposed interaction of exogenous siderophores with IROMPS which have yet to be assigned function, it has been proposed that

some siderophore-like molecules may rely on the porin protein OprF (Meyer, 1992). OprF is a major uptake route for many hydrophilic molecules across the outer membrane of *P. aeruginosa* (Nikaido and Hancock, 1986). Mutants deficient in OprF expression were observed to be very poor users of several exogenous siderophores (Meyer, 1992).

One particularly well documented example of *P. aeruginosa* utilising exogenous siderophores is that of the *E. coli* siderophore, enterobactin. Poole *et al.* (1990) demonstrated that EDDHA-induced growth inhibition could be reversed by addition of enterobactin. The presence of enterobactin induced expression of a novel 80 kDa IROMP termed PfeA. PfeA was found to have 60% homology with the *E. coli* enterobactin receptor FepA and shared similar immunological reactivities (Dean and Poole, 1993). However, mutants lacking PfeA still underwent enterobactin dependent growth, albeit at much higher concentrations of iron, suggesting two systems may be operating. One system appears to be of high affinity and is specifically inducible in the presence of enterobactin in iron-limiting growth conditions with the other being of much lower affinity and is independent of enterobactin for induction. Like pyoverdine, it was not possible to isolate a mutant that was completely devoid of ferrienterobactin transport reinforcing the notion that two systems exist to reduce the chance of a deleterious mutation occurring.

Iron transport has also been observed using 2,3-dihydroxybenzoic acid, a biosynthetic precursor of enterobactin, and N-(2,3-hydroxybenzoyl)-L-serine, an enterobactin breakdown product (Screen *et al.*, 1995). Iron transport using N-(2,3-

hydroxybenzoyl)-L-serine was energy-dependent and iron-repressible and growth of the bacteria in the presence of either pyoverdine or pyochelin had little effect. However, growth in the presence of enterobactin did result in an increased rate of iron transport *via* the inducible enterobactin uptake system. However, with 2,3-dihydroxybenzoic acid, iron transport was neither iron repressible nor strongly energy-dependent, hence a second system may be in use that is as yet uncharacterised.

1.8. Inositol Phosphate-Mediated Iron Transport in *Pseudomonas aeruginosa*

One compound recently discovered to have siderophore activity in *P. aeruginosa* is *myo*-inositol hexakisphosphate (*myo*-$InsP_6$ or phytic acid). Smith *et al.* (1994) demonstrated that *myo*-$InsP_6$ had siderophore activity and was able to reverse the iron-restricted growth inhibition of *P. aeruginosa* by EDDHA. ^{55}Fe-$InsP_6$ uptake was strongly iron-regulated and repressed after growth in iron-sufficient conditions. Using electron transport chain inhibitors, uptake of *myo*-$InsP_6$ was shown to be an active process. This appears to be the first report of *myo*-$InsP_6$ activity in prokaryotic cells.

1.8.1. Nomenclature and Stereochemistry of Inositol Derivatives

Inositol was first described as an optically inactive isomer of cyclohexane hexol which was designated "Inosit". Following the discovery and synthesis of other isomers, this was extended to "inositol" and there are now nine possible isomeric inositols, including one enantiomeric pair with the prefixes *scyllo-*, *myo-*, *neo-*, *epi-*,

cis-, *muco*-, *allo*-, D-*chiro*-(+) and L-*chiro*-(-) (reviewed by Potter and Lampe, 1995).

1.8.2. *myo*-Inositol

myo-Inositol is a *meso*-cylcohexane hexol, has a plane of symmetry and when in dilute solution at neutral pH, has five equatorial hydroxyl groups and one axial hydroxyl group. The carbon bearing the axial hydroxyl group is designated C-2 and the other carbons may be numbered from C-1 to C-6 starting on either side of C-2 and proceeding around the ring in either a clockwise or anticlockwise manner. By convention, numbering anti-clockwise in an assymetrically substituted inositol leads to a configerational D-prefix and clockwise numbering gives a substituted inositol with an L-pefix (Potter and Lampe, 1995).

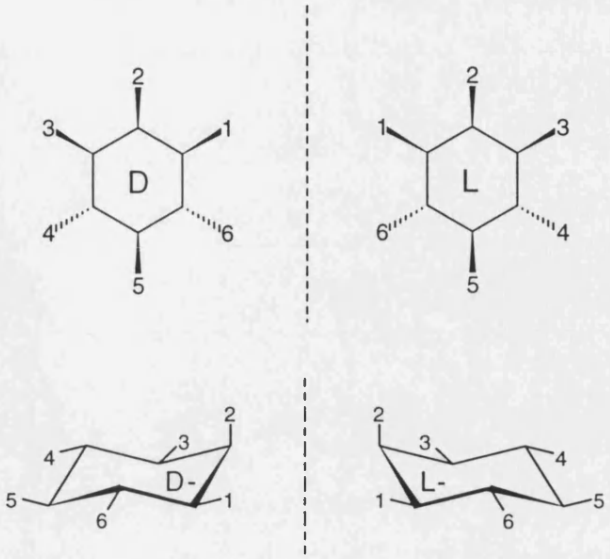


Figure 1.6. Diagrammatic representation of the numbering and stereo-chemistry of the inositol ring

Humans obtain the majority of *myo*-inositol in the diet from plants with only a small amount being biosynthesised. In both plants and animals, *de-novo* synthesis occurs by isomerisation of D-glucose 6-phosphate catalysed by L-*myo*-inositol-1-phosphate synthase. L-*myo*-inositol-1-phosphate synthase has been purified from yeast (Donahue and Henry, 1981), mammalian testes (Maeda and Eisenberg, 1980; Mauck *et al.*, 1980), and brain (Einsberg and Maeda, 1985). *myo*-Inositol is subsequently generated by the activity of inositol monophosphatase on L-*myo*-inositol-1-phosphate. This enzyme has also been purified from brain (Takimoto *et al.*, 1985; Attwood *et al.*, 1988; Meek *et al.*, 1988). The observation that the brain does not have access to dietary *myo*-inositol because of its inability to traverse the blood-brain

barrier, provides evidence that *de novo* D-glucose-6-phosphate-mediated *myo*-inositol biosynthesis must be in operation.

1.8.3. Inositol Polyphosphates in Eukaryotic Systems

Inositol polyphosphates are perhaps best known for their role as second messengers in eukaryotic cells. Interest in these important biological molecules dates back nearly 150 years when the parent compound, inositol was first isolated (reviewed by Potter and Lampe 1995). There was some interest in these compounds during the late 1950s although it was in 1975, when Michell (1975) proposed a link between agonist-stimulated phospholipid turnover and increased levels of intracellular calcium. The missing link was identified by Berridge and his co-workers as a *myo*-inositol polyphosphate second messenger (Streb *et al.*, 1983).

Cells communicate *via* hormones and neurotransmitters. Lipophilic hormones such as steroids can pass through the lipid bilayer of cell membranes and interact with specific intra-cellular targets. However, many hydrophilic chemical messengers are unable to traverse cell membranes and cannot interact with intra-cellular targets. Such compounds deliver their message by binding to specific receptors on the cell surface which in turn activate mechanisms that transmit the signal into the cell (Berridge and Irvine, 1989). This forms the basis of second messenger signalling whereby second messengers are employed to transmit the chemical message to the interior of the cell.

Briefly, the second messenger system comprises a ligand, such as a hormone, binding to an appropriate receptor, stimulating a GTP-binding protein (G-protein) to bind GTP. Activation of the G-protein results either in the stimulation or inhibition of other membrane-bound enzymes which act as amplifiers. Such amplifiers include K^+ channels, Ca^{2+} channels, adenylate cyclase (AC), guanylate cyclase (GC) and phosphatidylinositol-4,5-bisphosphate-specific phospholipase C (PtdIns-PLC), which in turn generate second messengers on the cytosolic side of the cellular membrane. Second messengers include adenosine 3'5'-cylic adenosine monophosphate (cAMP), 1,2-di-*O*-acylglycerol (DAG), Ins(1,4,5)P_3 and Ca^{2+} ions.

1.8.4. The Phosphatidylinositol Cycle

Phosphatides containing inositol comprise less than 10% of the total phospholipid in animal cells. Phosphatidylinositol (PtdIns) is the most common, forming over 90% of the total amount and is located primarily in the endoplasmic reticulum. The phosphoinositides PtdIns(4)P and PtdIns(4,5)P_2 are located primarily in the inner leaflet of the plasma membrane. Following activation, the receptor-associated G-protein activates the membrane bound phosophodiesterase (phosphoinositidase), phospholipase-C (Cockroft and Thomas, 1992). Phospholipase-C cleaves PtdIns(4,5)P_2 into two second messengers, D-*myo*-inositol 1,4,5 trisphosphate (*myo*-Ins(1,4,5)P_3) and 1,2-di-O-acylglycerol (DAG), which together form a bifurcating signalling pathway. *myo*-Ins(1,4,5)P_3 is hydrophilic and diffuses into the cytosol and activates the receptor of the Ca^{2+} channel on the endoplasmic reticulum resulting in release of calcium from internal stores. Raised cytosolic Ca^{2+} levels trigger processes including smooth muscle contraction, glycogen breakdown and exocytosis.

DAG activates protein kinase C (PKC) which phosphorylates serine and threonine residues in target proteins and acts synergistically with *myo*-Ins(1,4,5)P_3.

After eliciting the required response, *myo*-Ins(1,4,5)P_3 must be deactivated to terminate its action. Metabolism of *myo*-Ins(1,4,5)P_3 to free inositol is complex and many inositol phosphate intermediates are formed. The sequence of events is summarised below.

The action of a 5-phosphatase (Downes *et al.*, 1982) results in the production of *myo*-Ins(1,4)P_2 which, having no known second messenger role, causes termination of the calcium mobilising signal. Alternatively, a 3-kinase phosphorylates *myo*-Ins(1,4,5)P_3 to produce *myo*-Ins(1,3,4,5)P_4. Both *myo*-Ins(1,3,4,5)P_4 and *myo*-Ins(1,3,4)P_3, which is produced after the sequential action of the 5-phosphatase, may have moderate second messenger activity (Irvine, 1992). *myo*-Ins(1,3,4)P_3 is metabolised to *myo*-Ins(1,3)P_2 and/or *myo*-Ins(3,4)P_2 and then subsebsequently to Ins(1)P and Ins(3)P, respectively, which are finally dephosphorylated to inositol by the action of inositol monophosphatase.

Other metabolic pathways for these compounds are also becoming apparent. The action of a 5/6-kinase (Shears *et al.*, 1987; Balla *et al.*, 1987) phosphorylates *myo*-Ins(1,3,4)P_3 to *myo*-Ins(1,3,4,6)P_4 which has a moderate calcium mobilising ability (Gawler *et al.*, 1991; Ivorra *et al.*, 1991), and to *myo*-Ins(1,3,4,5)P_4. In animals, further action of the 5/6-kinase produces *myo*-Ins(1,3,4,5,6)P_5 which is in later metabolised to *myo*-InsP_6, *myo*-Ins(3,4,5,6)P_4 or *myo*-Ins(1,4,5,6)P_4.

1.8.5. Inositol Hexa- and Pentakisphosphates

myo-$InsP_6$ is the most abundant organic source of phosphate and is found in soil and all eukaryotic cells (Cosgrove, 1980). It has been found at levels up to an estimated 600 μM (Martin *et al.*, 1987) and whilst the exact biological role is unclear, *myo*-$InsP_6$ may serve as either a phosphate or inositol store as it is metabolised to other inositol phosphates. In addition, it has been reported to function as a neurotransmitter (Vallejo *et al.*, 1987), an antioxidant (Graf and Eaton, 1990) and may even have a role in the prevention and treatment of a range of cancers (Shamsuddin, 1995). Recently, *myo*-$InsP_6$ has been characterised with respect to its ability to prime human neutrophils hence having a pro-inflammatory role (Kitchen *et al.*, 1996). Many plant materials are rich in *myo*-$InsP_6$ where it accumulates particularly in seeds. Consequently, diets rich in plant-based products contain large amounts of *myo*-$InsP_6$ which has been the source of concern as the metal chelating ability of *myo*-$InsP_6$ may result in reduced absorption of many of the trace metals. The antioxidant effect is thought to result from the metal complexation properties of *myo*-$InsP_6$. The ability of *myo*-$InsP_6$ to complex Fe(III) is extremely high with an affinity constant estimated to be between 10^{25}-10^{30} M^{-1} (Poyner *et al.*, 1993). Graf *et al.* (1984) proposed that the ability of *myo*-$InsP_6$ to complex Fe(III) prevents $HO^{\bullet}$ formation from the superoxide anion *via* the iron-catalysed Haber-Weiss redox cycle. In addition, Graf *et al.*, (1984) suggested that *myo*-$InsP_6$ increased oxygen-mediated oxidation of Fe(II) whilst blocking the hydrogen peroxide-mediated oxidation and concomittant hydroxyl radical production.

The discovery of intracellular inositol pentakisphosphates brought further understanding of the iron binding properties of *myo*-$InsP_6$. All bind Fe(III) although only those containing 1,2,3 (equatorial-axial-equatorial) trisphosphate grouping have antioxidant properties (Hawkins *et al.*, 1993). The major $InsP_5$ is Ins(1,3,4,5,6)P_5 which is also found in the majority of mammalian cells. Consequently, *myo*-$InsP_6$ and Ins(1,3,4,5,6)P_5 are present in larger amounts than all other inositol polyphosphates (Berridge and Irvine, 1989; Shears, 1992). Ins(1,3,4,5,6)P_5 is known to regulate the affinity of avian haemoglobin for oxygen and in common with *myo*-$InsP_6$ is thought to have some function as a neurotransmitter (Vallejo *et al.*, 1987).

The intracellular levels of *myo*-$InsP_6$ and *myo*-Ins(1,3,4,5,6)P_5 fluctuate slowly in contrast to inositol which is incorporated rapidly into inositol lipids and *myo*-Ins(1,4,5)P_3 (French *et* al., 1991; Menniti *et* al., 1990; Oliver *et* al., 1992). *De novo* synthesis of *myo*-$InsP_6$ and *myo*-Ins(1,3,4,5,6)P_5 proceed very slowly with *myo*-Ins(1,3,4,5,6)P_5 formed by slow phosphorylation of *myo*-Ins(1,3,4)P_3 (Hughes *et al.*, 1989; Balla *et al.*, 1989) by the actions of the 5/6-kinase. Even slower is the phosphorylation of *myo*-Ins(1,3,4,5,6)P_5 to *myo*-$InsP_6$ (Stephens *et al.*, 1991). In contrast, the levels of *myo*-Ins(1,4,5)P_3 fluctuate several fold within seconds, hence *myo*-$InsP_6$ and *myo*-Ins(1,3,4,5,6)P_5 are not regarded as participating in general short-term regulatory processes. Consequently, their roles in phosphate and inositol storage and as antioxidants appear more likely.

1.9. Aims and Objectives

P. aeruginosa is a particularly resistant and robust pathogen reponsible for life threatening infections in certain groups of patients. Due to the failure of many currently available antimicrobial agents, improving our understanding of the methods of iron acquisition in *P. aeruginosa* may be useful in developing new modes of treatment directed against this organism.

myo-Inositol hexakisphosphate was first reported to have siderophore activity in *P. aeruginosa* PAO1 by Smith *et al.*, (1994) where it was able to reverse growth inhibition by EDDHA. The aim of this study was to characterise further this phenomenon and identify the mechanism by which *myo*-$InsP_6$ transport is mediated.

To achieve this, attempts were made to identify key structural motifs responsible for *myo*-$InsP_6$-mediated iron uptake. This was achieved by examining structure-iron transport relationships of a wide range of inositol penta-, tetra-, and trisphosphates. The next aim was to determine whether a correlation existed between the ability of these compounds to mediate iron uptake into *P. aeruginosa* PAO1 and their ability to interact with ferric iron. It was then aimed to identify the mechanisms associated with release of iron from the inositol phosphates to the bacterium. In addition, it was attempted to identify the mechanisms associated with *P. aeruginosa* PAO1 that afforded inositol phosphates the ability to mediate iron transport into the bacterium. These were achieved by examing the possible roles of enzyme systems, the Gram-negative outer membrane with its associated proteins and the dependence upon

active transport systems. Finally, attempts were made to isolate a mutant of *P. aeruginosa* PAO1 that was unable to use ferri-inositol phosphates as a source of iron.

Chapter 2

Materials

2.1. Bacterial Strains

Organism	Characteristics	Source
***P. aeruginosa* PAO1**	Wild Type	Hancock and Carey, 1979
***P. aeruginosa* IA1**	Pvd-, Pch- mutant of *P. aeruginosa* PAO1	Ankenbauer *et al.*, 1985
***P. aeruginosa* K372**	Pch-, derivative of *P. aeruginosa* PAO6609 (Pvd-), met-.	Poole *et al.*, 1991
***P. aeruginosa* K239**	*P. aeruginosa* PAO1 (pMT1000::Tn*501*)	Poole and Hancock, 1986
***P. aeruginosa* PH2**	*P. aeruginosa* IA1, Str^R	This study
***P. aeruginosa* PH3**	*P. aeruginosa* PH2 (pMT1000::Tn*501*)	This study
***P. aeruginosa* PH4**	*P. aeruginosa* K372 (pMT6121::Tn*1737*KH)	This study
***P. aeruginosa* H729**	OprD::Ω, Km^R, met-.	Trias *et al.*, 1989
***P. aeruginosa* H636**	OprF::Ω, Str^R, met-.	Woodruff and Hancock, 1988
***P. aeruginosa* H576**	OprP::Ω, Hg^R, met-.	Poole and Hancock, 1986
***E. coli* CT725**	*E. coli* DH1 (pMT6121::Tn*1737*KH)	Tsuda et. al., 1995

Table 2.1. Bacterial Strains.

2.2. Chemicals

All chemicals were supplied by Fisons (Loughborough, U.K.) and Sigma Chemical Co. (Poole, U.K.) unless stated differently. Chemicals were of Analytical Reagent Grade or equivalent.

2.3. Inositol Phosphates

myo-InsP_6 and *myo*-Ins(1,4,5)P_3 were obtained from Sigma Chemical Co. (Poole, U.K). *myo*-Ins(1,3,4,6)P_4 was obtained from Calbiochem (San Diego, Ca, USA) and *myo*-Ins(1,2,3)P_3 was obtained from Dr. I. Spiers (Department of Pharmaceutical and Biological Sciences, Aston University) All other inositol phosphates were from Prof. B.V.L. Potter, Dr. S.J. Mills, Dr. D. Lampe and Dr. A. Riley (School of Pharmacy and Pharmacology, University of Bath).

2.4. ^{55}Fe Transport Assay Glassware

Glassware for iron transport assays was soaked overnight in 0.01% EDTA solution and rinsed thoroughly in Milli-Q water prior to sterilisation to reduce levels of contaminating iron.

2.5. Radiochemicals

Iron-55 ferric chloride in aqueous solution, 740 MBq/ml, 1167 MBq/mg. E.I. DuPont de Nemours & Co. Ltd. (Wilmington, De, USA).

[^{3}H]-*myo*-Inositol hexakisphosphate, 0.37 MBq/ml, 119 Mbq/mg. E.I. DuPont de Nemours & Co. Ltd. (Wilmington, De, USA).

[^{3}H]-D-*myo*-Inositol (1,4,5) Trisphosphate, 0.37 Mbq/ml, 1.35 Mbq/mg. Amersham Life Science Inc. (Cleveland, Oh, USA).

2.6. Complex Media

Luria Broth	Bacto tryptone	10 g
	Yeast extract	5 g
	NaCl	5 g
	ddH_2O	to 1000 ml
	pH	7.5.
Luria Agar	Luria Broth + 1.5% Agar (Difco).	
Nutrient Agar	Nutrient agar (Oxoid)	28 g
	ddH_2O	to 1,000 ml.

2.7. Chemically Defined Medium (Meyer and Abdallah, 1978)

Succinate Medium	K_2HPO_4	6 g
	KH_2PO_4	3 g
	$(NH_4)_2SO_4$	1 g
	$MgSO_4.7H_2O$	0.2 g
	Sodium succinate	4 g
	ddH_2O	to 1,000 ml
	pH	to 7.0.

For growth of *P. aeruginosa* K372 and *P. aeruginosa* PH4, methionine was added to a final concentration 1 mM.

2.8. Equipment

Automatic pipettes: Gilson pipetman, P-2, P-20, P-200 and P-1000 (Anachem Ltd., Luton, U.K.). Finnpipette Digital 10 ml (Labsystems, Helsinki, Finland).

Balances: Mettler AE 50 (Mettler Instrumente AG, Zurich, Switzerland). Oertling TP 31 (Oertling, U.K.).

Centrifuges: MSE Micro Centaur microfuge (Measuring and Scientific Equipment Ltd., London, U.K.). Beckman J2-MC High Speed Centrifuge using JA-20.1 and JA-14 rotors. Beckman L8-M Ultracentrifuge using a Ti-70 rotor (Beckman Instruments Inc., Palo Alto, Ca, USA). Sorvall OTD Ultracentrifuge using a TH-641 Swinging Bucket Ultraspeed Rotor (DuPont Company, Medical Products, Sorvall Instruments, Wilmington, De, U.S.A.).

Filtration Equipment: 25 mm Swinnex units (Millipore, Molsheim, France). Whatman 25 mm Cellulose Nitrate 0.2 μm Membrane Filters (Whatman, Maidstone, U.K.).

Gel Electrophoresis Apparatus: Bio-Rad Mini Protean II Electrophoresis System (Bio-Rad Laboratories Ltd., Richmond, Ca, U.S.A.).

Heated Water Bath: Grant JB2 (Grant Instruments (Cambridge) Ltd., Cambridge, U.K.).

Heating Block: Techne Dri-Block DB-2A (Techne (Cambridge) Ltd., Duxford, U.K.).

Incubators: New Brunswick Scientific Controlled Environment Incubator Shaker (New Brunswick Scientific, Edison, NY, USA) and Heraeus B6060 (Heraeus Equipment Ltd., Brentwood, U.K.).

Magnetic Stirrer: Stuart Magnetic Stirrer (Stuart, U.K.)

pH Meter: Corning pH Meter 240 (Ciba Corning Diagnostics Ltd., Watford, U.K.).

Power Supply: Bio-Rad 200/2.0 Power Supply (Bio-Rad Laboratories Ltd., Watford, U.K.)

Rotary Evaporator: Rotavapor-R (Buchi, Flawil, Switzerland).

Scintillation Counter: LKB Wallac 1215 RackBeta Liquid Scintillation Counter (Wallac, Finland).

Sonicator: MSE 150 Watt Ultrasonic Disintegrator Mark 2. (Measuring and Scientific Equipment Ltd., London, U.K.).

Sonicating Water Bath: Sonicleaner Type 6442AE (Ultrasonics Ltd., U.K.)

Spectrophotometer: Milton Roy Spectronic 601 (Milton Roy, Stone, U.K.).

Spectrophotometer Cuvettes: Elkay Micro Square Ultra-Vu Disposable Cuvettes (Elkay Products Inc., Ma, USA).

Water Supply: Milli-Q Plus P.F. (Millipore, Molsheim, France)

Whirlimixer: Fisons Whirlimixer WM/250/SC/P (Fisons, Loughborough).

Chapter 3

Experimental Methods

3.1. Measurement of Bacterial Cell Concentration in Liquid Media

Bacterial cell concentrations in liquid media were quantified using spectrophotometric techniques. At low cell concentrations, the light scattered by a bacterial suspension is directly proportional to the cell concentration in accordance with the Beer-Lambert Law:-

$$OD = \text{Log } (I_o/I)$$

Where OD = optical density

I_o = intensity of incident light.

I = intensity of emergent light.

This relationship is obeyed between OD 0.03 and 0.3. Measurements of bacterial cell suspensions were made at 470 nm (OD_{470}) to maximise scattering of light by the cells whilst minimising absorption by other media components and cell products. An OD_{470} of 1.0 indicates an approximate cell concentration of $1x10^9$ cells/ml.

3.2. Freezing Bacterial Samples

Cells were grown overnight on nutrient agar supplemented with appropriate antibiotics. 1.5 ml LB+50% glycerol was placed on the NA surface and the cells suspended using a sterile glass spreader. Samples were stored at -70°C.

3.3. Preparation of *Pseudomonas aeruginosa* Membrane Fraction

One litre of overnight *P. aeruginosa* culture grown at 37°C in a shaking orbital incubator was harvested by centrifugation at 6,000 g for 10 minutes at 4°C. The cells were washed with 50 ml 0.9% saline solution, centrifuged as before, then suspended in 20 ml 0.9% saline. Cells were lysed by sonication in an ice bath by 10 x 30 s pulses with 30 s intervals to allow cooling.

The sonicated suspension was centrifuged at 5,000 g for 5 minutes at 4°C to remove unbroken cells. The supernatant was centrifuged at 20,000 g for 1 hour and the membrane pellet washed and suspended in sterile Milli-Q water. The protein content was determined by the Lowry assay (Lowry *et al.*, 1951), and the suspension stored at -20°C (section 3.6).

3.4. Preparation of *Pseudomonas aeruginosa* Outer Membranes Using the Sarkosyl Method

The method used was based on that by Filip *et al.*, (1973). Cells grown overnight at 37°C in an orbital shaker were harvested by centrifugation at 6,000 g for 10 min at 4°C. The supernatant was discarded, the cells washed with 50 ml 0.9% saline solution and resuspended in 20 ml 0.9% saline. Cells were lysed by sonication in an ice bath by 10 x 30 second pulses with 30 second intervals to allow cooling.

The sonicated suspension was centrifuged at 6,000 g for 5 min at 4°C to remove unbroken cells. The supernatant was transferred to centrifuge tubes and Sarkosyl (N-lauroyl-sarcosinate) solution (20%w/v) added to a final concentration of 2%. After 1 h,

the mixture was centrifuged for 60 min at 20,000 g and 4°C. The resulting pellet was resuspended in sterile Milli-Q water and stored at -20°C The protein content was determined using the Lowry assay (section 3.6; Lowry *et al.*, 1951).

3.5. Sucrose Gradient Preparation of Cytoplasmic and Outer Membranes of *Pseudomonas aeruginosa*

Cells harvested from a 3 l *P. aeruginosa* overnight culture were washed with 0.9% saline, suspended in 20 ml 0.9% saline and broken by sonication as described in section 3.4. Following centrifugation at 5,000 g to remove unbroken cells, membranes were pelleted by centrifugation at 35,000 g for 1 h at 4°C and suspended in 1 ml 10% w/v sucrose solution. Sucrose gradients were prepared in centrifuge tubes by gently layering sucrose solutions of varying strength, with the strongest sucrose concentration at the bottom of the centrifuge tube. Solutions used were 1.2 ml each of 65%, 60%, 55%, 50%, 45%, 40% and 35% w/v sucrose and the gradients stored at 4°C for one hour before use. The membrane sample was added to the top of the gradient and the gradients centrifuged for 18 h at 35,000 rpm and 4°C using a Sorvall TH-641 swing-out rotor. The lighter cytoplasmic membranes form a band near the top of the gradient whilst the heavier outer membranes partition towards the bottom of the gradient.

Following centrifugation, gradients were removed by transferring 200 µl samples from the top of the gradients to 96 well microtitre plates. Aliquots containing membrane fractions were identified using a plate reader measuring at 340 nm and the outer membrane fractions pooled. Membranes were pelleted from the sucrose solution by addition of sterile distilled water and centrifuging at 50,000 g for 40 minutes at 4°C. The

membrane pellets were washed and suspended in 1 ml sterile distilled water and stored at -20°C.

3.6. Protein Quantification Using the Lowry Assay

The Lowry protein assay was used to determine the protein content of outer membrane preparations (Lowry *et al.*, 1951). A standard curve was obtained using dilutions of a 1 mg/ml stock solution of bovine serum albumin (BSA).

OMPs were analysed by adding 100 µl outer membrane suspension to 400 µl sterile Milli-Q water in a small boiling tube. A blank was prepared using 500 µl sterile Milli-Q water.

An equal volume (500 µl) of 0.5 M NaOH was added to each sample and the mixture heated to 100°C for 10 minutes. The mixtures were allowed to cool to room temperature followed by addition of 2.5 ml Lowry C reagent (1 ml Lowry B = 0.5% w/v $CuSO_4.5H_2O$ / 1% w/v NaK tartrate solution + 24 ml Lowry A = 5% w/v Na_2CO_3) with shaking. Folin and Ciocalteu's Phenol Reagent (500 µl) was added after 10 min and mixed by vortexing. Optical density at 750 nm (OD_{750}) of all samples was recorded after 30 minutes against the similarly treated water blank

3.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation of membrane proteins was performed by gel electrophoresis using a Mini-Protean system, according to the methods described by Lutenberg *et. al.*, (1975). The running and stacking gel were prepared as described (table 3.1), and polymerisation

initiated by addition of N,N,N',N'-tetramethylethylenediamine (TEMED). The running gel was poured between glass plates separated by 1.5 mm plastic spacers and allowed to set for 10 min. A spray of electrode buffer on top of the gel ensured complete polymerisation and formation of a straight edge. This buffer solution was removed and the stacking gel cast in a similar method. A Teflon comb was inserted between the plates to create wells for sample application.

Samples were denatured at 100°C for 10 min with an equal volume of sample buffer before loading into the gel. The electrode buffer contained 25 mM Tris, 190 mM glycine and 0.1% SDS. In the Mini-Protean system, a constant voltage of 80 V was applied whilst the sample ran through the stacking gel and was increased to 180 V for electrophoresis through the running gel. Electrophoresis continued until the tracking dye had migrated to within 5 mm of the bottom of the gel. Gels were stained for protein with 0.1% w/v Coomassie brilliant blue R-250 in 50% methanol /10% glacial acetic acid. Gels were subsequently destained in 10% methanol/20% acetic acid.

Pre-stained low range molecular weight markers (Bio-Rad) consisted of the following:-

Protein	**Molecular Weight**
Phosphorylase B	107,000
Bovine Serum Albumin	76,000
Ovalbumin	52,000
Carbonic Anhydrase	36,800
Soyabean Trypsin Inhibitor	27,200
Lysozyme	19,000

Constituent	Running Gel (10%)	Stacking Gel (5%)	Sample buffer
Acrylamide Soln.	3.92 ml	2.0 ml	
SDS 10% $^{w}/_{v}$	0.3 ml	0.12 ml	5 ml
1.5M Tris pH 8.8	3.7 ml		
0.5M Tris pH 6.8		3.0 ml	2.5 ml
Milli-Q Water	3.78 ml	6.4 ml	5 ml
10% TEMED Soln.	30 µl	32 µl	
AMPS 10% $^{w}/_{v}$	40 µl	40 µl	
Glycerol			2.5 ml
2-mercaptoethanol			0.25 ml
5% Bromophenol Blue			0.2 ml

Acrylamide solution: 30% Acrylamide/Bis Solution, 37.5:1 (Bio-Rad Laboratories Ltd., Watford, U.K.).

AMPS: Ammonium persulphate (freshly prepared).

Bis: N,N'-methylene-bis-acrylamide

TEMED: N,N,N,'N'-tetramethylethylene diamine

Table 3.1. Compositions of Running Gel, Stacking Gel and Sample Buffer for SDS-PAGE

3.8. Preparation of *Pseudomonas aeruginosa* Spheroplasts

The method used was based on that described by Booth and Curtis, (1977). Log-phase *P. aeruginosa* grown in 3 l succinate minimal medium was harvested by centrifugation at 8,000 g for 10 min and 4°C. Cells were suspended in 100 ml Tris/Sucrose solution (33 mM Tris pH 8.0, 0.25 M sucrose) and lysozyme and EDTA added to final concentrations of 30 µg/ml and 250 µg/ml, respectively. Following incubation at room temperature for 10 minutes, the cell suspension was centrifuged at 5,000 g for 5 min to

remove whole cells and the resulting supernatant centrifuged at 10,000 g for 30 min to pellet the spheroplasts gently. Spheroplasts were carefully resuspended in 10 ml tris/sucrose and used immediately for iron transport assays.

The spheroplast:whole cell ratio was determined by dilution of cells in either sterile Milli-Q water or sterile tris/sucrose, followed by viable counting. Cells surviving dilution in water were judged to be whole cells, whereas dilution in Tris/sucrose represented the combination of spheroplasts and whole cells.

3.9. ^{55}Fe Transport Assays Using Whole Cells or Spheroplasts

Glassware for iron transport assays was treated as described in section 2.4. Starter cultures were incubated for 8 h and sub-cultured (1:10) for a further 17 h.

Whole cells grown in succinate medium in an orbital incubator at 37°C and 300 rpm were harvested by centrifugation at 5,000 g and 4°C for 20 min. The cells were washed twice in 100 mM MOPS buffer (pH 7.0) and suspended to an OD_{470} of 2.0 in MOPS buffer (pH 7.0) and 60 μM glucose to a final volume of 500 μl. The cell suspension was equilibrated at 37°C for 15 min prior to transport studies.

The ^{55}Fe-inositol phosphate complexes were formed at 37°C 15 min prior to uptake as 200 μM inositol phosphate and 400 nM [^{55}Fe]-$FeCl_3$ in MOPS buffer (pH 7.0) to a final volume of 500 μl. Addition of the ^{55}Fe-inositol phosphate mixture to the cell suspension resulted in final concentrations of 100 μM inositol phosphate and 200 nM $^{55}FeCl_3$ (500:1 ratio). The resulting mixture was incubated at 37°C and iron transport assays

performed by withdrawing 50 µl samples and filtering through 0.2 µm nitrocellulose filters. The filters were washed with 20 ml 0.9% saline solution and air dried. When dry, filters were transferred to 4 ml scintillation fluid (Optiphase Hisafe, LKB) and the cell-associated activity retained on the filter determined by scintillation counting using the ^{3}H channel.

When performing ^{55}Fe transport assays using spheroplasts, MOPS buffer and 0.9% saline were replaced by Tris/sucrose solution (section 3.8) to offer osmotic protection. All other aspects of the assay were performed as above.

3.10. Hydroxyl Radical Assay

The method used was based on that described by Graf *et. al.*, (1984) and adapted by Hawkins *et al.*, (1993). The assay mixture was prepared as follows to a final volume of 1 ml:-

50 µl 1 M DMSO
100 µl 0.02% hypoxanthine in 20 mM tris pH 7.4
50 µl xanthine oxidase (0.8 units/ml)
10 µl 10 mM inositol phosphate
10 µl 500 µM $FeCl_3$ (freshly prepared)
780 µl 20 mM tris pH 7.4.

The reaction mixture was incubated at 37°C for 30 min. Two hundred and fifty µl reagent B was added (15 g $NH_4O_2CCH_3$, 0.3 ml glacial acetic acid, 0.2 ml acetyl acetone to 100 ml with distilled water) and the mixture heated at 60°C for 15 min. The

generation of yellow colouration was measured by reading the absorbance at 410 nm using distilled water as a blank.

3.11. *Pseudomonas aeruginosa* PAO1 Reductase Assay

The method used was that described by Halle and Meyer (1992a) and is based on the spectrophotometric measurement at 562 nm of the Fe(II)-Ferrozine complex formed during the assay.

Four l of overnight *P. aeruginosa* culture grown in succinate minimal medium were harvested by centrifugation at 6,000 g and 4°C. Cells were washed twice with distilled water, resuspended in 25 ml buffer A (50 mM Tris, 0.1 M KCl at pH 7.4) and broken by sonication (10 x 30 s pulses with 1 min intervals for cooling). The protein content of the bacterial lysate was assayed using the Lowry Protein Assay (Lowry *et. al.*, 1951) using BSA as a standard (Section 3.6.) and adjusted to 0.8 mg/ml by addition of buffer A.

Reductase assays were performed in a glass spectophotometer cuvette as follows. Two millilitres bacterial lysate (0.8 mg/ml) were added to the cuvette followed by addition of inositol phosphate and $FeCl_3$, each to a final concentration of 0.2 mM. Anaerobiosis was achieved by flushing the mixture with argon for 10 min. The reaction was started by addition of FMN, Ferrozine and NADH to final concentrations of 0.05 mM, 0.8 mM and 0.15 mM, respectively. The cuvette was transferred to the spectrophotometer and the reaction followed by measuring the absorbance at 562 nm. A continous stream of argon was maintained through the reaction mixture for the duration of the reaction.

The assay was performed at room temperature, all stock solutions were prepared in buffer B (25 mM Tris, pH 7.4) and both the bacterial lysate and stock solutions were stored at -20°C without loss of activity over a one month period. The amount of Fe(II)-Ferrozine produced was calculated from the known extiction coefficient for Fe(II)-Ferrozine (28,000 $M^{-1}cm^{-1}$).

3.12. Competitive Binding Assays of [^{3}H]-$InsP_6$ to *Pseudomonas aeruginosa* Membranes

Membranes were homogenised immediately before use in 100 mM HEPES buffer at 4°C to a final concentration of 1.0 mg/ml. Binding was performed at 4°C for one hour in a 1.5 ml microcentrifuge tube using 1 mg homogenised membrane suspension and [^{3}H]-$InsP_6$ at a final concentration of 1.0 nM.

Free ligand was separated from bound ligand by centrifugation at 13,000 g for 5 min. Membrane pellets were washed twice with water at 30°C, air dried and solubilised overnight by addition of 100 µl Soluene (Packard, Meriden, Ct, U.S.A). One ml scintillation cocktail was added and the remaining activity determined by scintillation counting using the ^{3}H channel.

3.13. Isolation and Purification of Pyoverdine

The method used was based on that described by Poole *et al.* (1991). An overnight culture of *P. aeruginosa* grown in 500 ml succinate minimal medium was harvested by centrifugation at 8,000 g for 10 minutes at 4°C. The supernatants were collected and the

liquid phase removed using a rotary evaporator. The residual material was resuspended in 12 ml sterile Milli-Q water, centrifuged at 16,000 g to remove insolubles, and extracted once with an equal volume of ethyl acetate. The organic phase was discarded and solid NaCl added to the aqueous phase to saturation. The aqueous phase was extracted twice with 0.5 volume phenol:chloroform and the organic phases pooled. Following addition of 2 volumes diethyl ether to the organic phase, the precipitated pyoverdine was pelleted by centrifugation at 10,000 g for 10 min at 4°C, and the resulting pellet washed three times with 3 ml diethyl ether. The pellet was air-dried, suspended in double distilled water to 20 mg/ml, and stored at -20°C.

3.14. Transposon Insertion Mutagenesis in *Pseudomonas aeruginosa*

Two strains were subjected to transposon mutagenesis, *P. aeruginosa* IA1 using pMT1000(Tn*501*) and *P. aeruginosa* K372 using pMT6121 (Tn*1737*KH).

3.14.1. Tn*501* Insertion Mutagenesis of *Pseudomonas aeruginosa* IA1

Tn*501* was obtained in pMT1000, an R68 plasmid derivative (Poole and Hancock, 1986), which is temperature-sensitive for replication and maintenance. pMT1000 was supplied in *P. aeruginosa* K239 (*P. aeruginosa* PAO1 containing pMT1000) and was transferred to *P. aeruginosa* IA1 by conjugation. Selection against K239 was achieved using a spontaneous streptomycin resistant variant of *P. aeruginosa* IA1 (*P. aeruginosa* PH2).

3.14.1.1. Spontaneous *Pseudomonas aeruginosa* IA1 Streptomycin-resistant Mutant

A spontaneous streptomycin resistant variant of *P. aeruginosa* IA1 was isolated using a method based on that described by Tsuda and Iino (1983). One hundred µl IA1 suspension at $1x10^4$ cells/ml was plated onto LA+Str^{750}. Plates were incubated at 37°C for 48 hours and a spontaneous mutant isolated termed *P. aeruginosa* PH2.

3.14.1.2 Conjugation of *Pseudomonas aeruginosa* K239(pMT1000::Tn*501*) with *Pseudomonas aeruginosa* PH2

P. aeruginosa K239(pMT1000::Tn*501*), the donor strain, was incubated overnight with shaking at 30°C in LB in the absence of antibiotics to prevent carry-over into the conjugation mix. *P. aeruginosa* PH2, the recipient strain, was incubated overnight with shaking at 37°C. Fifty microlitres of donor and recipient cultures were spotted onto LA plates, incubated overnight at 30°C and the resulting cells harvested using 3 ml LB. Cells were plated at various dilutions on LA+Hg^{15}+Str^{750} and incubated overnight at 30°C. Transconjugants were isolated and grown in succinate minimal medium containing Hg^{15}+Str^{750} using K239 and PH2 as controls. Growth in the absence of siderophore production confirmed the transconjugants to be PH2(pMT1000::Tn*501*). An example of this strain was isolated and termed PH3.

3.14.1.3. Frequency of Transposition.

The frequency of transposition i.e. the extent of transposon movement between plasmid and chromosome, was calculated by viable counting. An overnight culture of *P. aeruginosa* PH3 was serially diluted and plated in duplicate onto LA plates which were incubated at 30°C and 42°C, respectively The viable counts at each temperature were

calculated. In addition, pMT1000 carries tetracycline resistance which, like mercury resistance, is temperature-dependent. Because tetracycline resistance is conferred by the vector and not Tn*501*, transformed colonies were tested for tetracycline sensitivity to confirm loss of the vector.

3.14.1.4. Tn*501* insertion mutagenesis of *Pseudomonas aeruginosa* PH3

Pseudomonas aeruginosa PH3 was grown overnight at 30°C, plated at various dilutions onto LA+Hg^{15} and incubated at 42°C for 24 h. The overnight culture was stored at 4°C. The remaining culture was plated onto LA+Hg^{15} to give approximately 400 cfu per plate when grown at 42°C. The resulting colonies were screened by picking onto selective media in the order:-

i) Succinate minimal agar
ii) Succinate minimal agar+ 500 μM *myo*-$InsP_6$
iii) Luria agar + 500 μM *myo*-$InsP_6$
iv) Luria agar + Hg^{15}

The selective plates were incubated for 48 hours at 37°C and examined for growth.

3.14.2. Tn*1737*KH Insertion Mutagenesis of *Pseudomonas aeruginosa* K372

This transposon mutagenesis system is based on the transposon Tn*1737*KH located in the plasmid pMT6121 and like Tn*501* carries mercury resistance for use as a selective marker. pMT6121 is also temperature sensitive capable of replication at 30°C but inhibited at 42°C.

3.14.2.1. Conjugation of *Escherichia coli* CT725 with *Pseudomonas aeruginosa* K372

pMT6121 containing Tn*1737*KH was transferred from *E. coli* CT725 to *P. aeruginosa* K372 by conjugation as described in section 3.13.1.2. K372(pMT6121::Tn*1737*KH) trans-conjugants were selected using Hg^{15}+Km^{250}. One such transconjugant was isolated and termed *P. aeruginosa* PH4. Confirmation that the new host was *P. aeruginosa* was achieved by adding 20 µl 10 % N,N,N',N'-Tetramethyl-p-phenylenediamine solution to cells smeared onto 3MM paper. The presence of a purple colouration confirmed the cells were oxidase-positive *P. aeruginosa* rather than the *E. coli* donor strain.

The frequency of transformation and tests regarding tetracycline sensitivity were performed as described previously (3.14.1.3.)

3.14.2.2. Tn*1737*KH Mutagenesis of *Pseudomonas aeruginosa* PH4

Mutagenesis of *P. aeruginosa* PH4 was performed using similar procedures as before with the exception that succinate minimal medium was always supplemented with 1 mM methionine since the parent strain, *P. aeruginosa* K372, is a methionine auxotroph. Consequently, the selective media were:-

i) Succinate minimal agar + 1 mM Met
ii) Succinate minimal agar+ 1 mM Met + 500 µM *myo*-$InsP_6$
iii) Luria agar + 500 µM *myo*-$InsP_6$
iv) Luria agar + Hg^{15}

Chapter 4

Inositol Phosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1

4.1. Introduction

myo-Inositol hexakisphosphate (*myo*-$InsP_6$) has been reported to have siderophore activity in *P. aeruginosa* (Smith *et al.*, 1994). It is found in the natural environment of *P. aeruginosa*, particularly soil, and is thought to occur in many, if not all, plant and animal cells (Cosgrove 1980; Stephens *et al.*, 1991). The siderophore activity of *myo*-$InsP_6$ was strongly iron-regulated, being repressed after growth in iron-replete conditions and was inhibited by electron transport chain inhibitors such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In this chapter, a structure-iron transport relationship is built up using a number of lower inositol polyphosphates.

4.2. *myo*-Inositol Hexakisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1

The ability of *myo*-$InsP_6$ to mediate iron transport into *P. aeruginosa* was determined under the conditions used throughout this study and was used as a standard against which all other compounds could be compared. The structure of *myo*-$InsP_6$ is illustrated below.

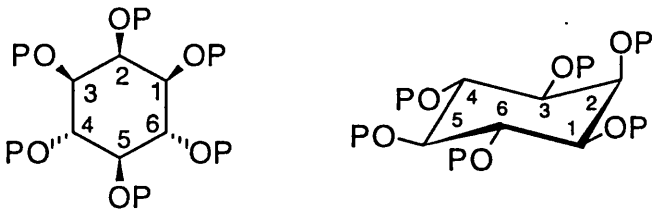


Figure 4.1. *myo*-Inositol Hexakisphosphate

The resulting *myo*-$InsP_6$-mediated iron transport into *P. aeruginosa* PAO1 is illustrated in figure 4.2 (p78); the initial rate of iron uptake was 1.63 pmol/min/10^9 cells and after 30 minutes the total amount accumulated was 19.08±1.59 pmol/10^9 cells (Mean±SEM, n=3).

4.3. Inositol Pentakisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1

Iron transport assays were performed using *myo*-Ins(1,3,4,5,6)P_5, D/L-*myo*-Ins(1,2,4,5,6)P_5, *myo*-Ins(1,2,3,4,6)P_5 and D/L-*myo*-Ins(1,2,3,4,5)P_5, and their structures are illustrated below.

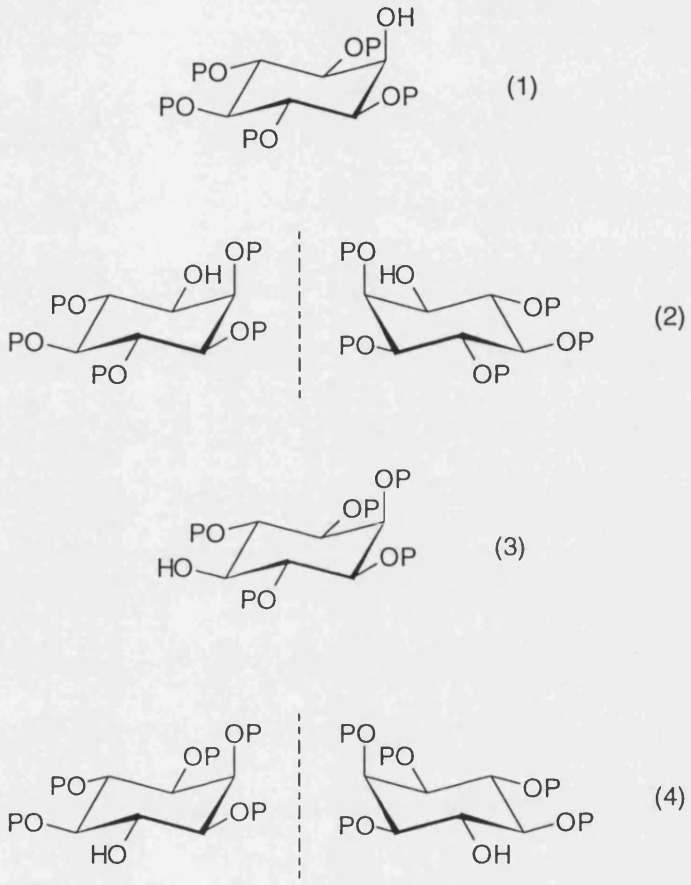


Figure 4.3. *myo*-Inositol Pentakisphosphates (1, *myo*-Ins(1,3,4,5,6)P_5; 2, D/L-*myo*-Ins(1,2,4,5,6)P_5; 3, *myo*-Ins(1,2,3,4,6)P_5; 4, D/L-*myo*-Ins(1,2,3,4,5)P_5).

The use of *myo*-InsP_5s allowed the determination of the effects of removing individual phosphate groups hence modifying several vicinal phosphate group

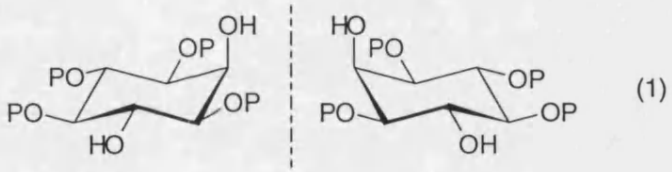
arrangements. The resulting *myo*-$InsP_5$-mediated iron transport profiles are illustrated in figure 4.4 (p79) and the data are summarised in table 4.1.

Inositol Polyphosphate	**Initial Rate of ^{55}FeUptake (pmol/min/10^9cells)**	**^{55}Fe Accumulated After 30 min (pmol/10^9 cells) (Mean±SEM, n=3)**
***myo*-Ins(1,3,4,5,6)P_5**	9.52	26.08±1.42*
D/L-*myo*-Ins(1,2,4,5,6)P_5	2.70	17.26±1.85
***myo*-Ins(1,2,3,4,6)P_5**	1.16	13.37±0.58
D/L-*myo*-Ins(1,2,3,4,5)P_5	0.73	9.18±1.15
***myo*-InsP_6**	1.63	19.08±1.59

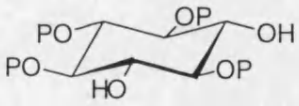
Table 4.1. *myo*-Inositol pentakisphosphate-mediated iron transport into *P. aeruginosa* PAO1. Uptake data from *myo*-$InsP_6$ is duplicated for comparison (* accumulated after 15 min).

4.4. Inositol Tetrakisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1

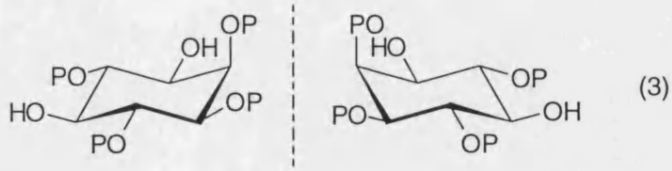
A range of inositol tetrakisphosphates were used to develop further the structure-iron transport relationships. Using these compounds it was possible to examine the importance of several bis- and trisphosphate motifs and whether the use of either D- or L- enantiomers had any effect on the ability to mediate iron transport into *P. aeruginosa*. Using these compounds, it was also possible to determine the effect of adding large functional groups to the inositol ring and whether changing the orientation of the 2-position functional groups from the axial to the equatorial orientation had any effect. The compounds used are illustrated below.



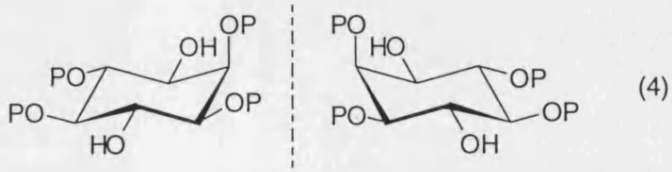
(1)



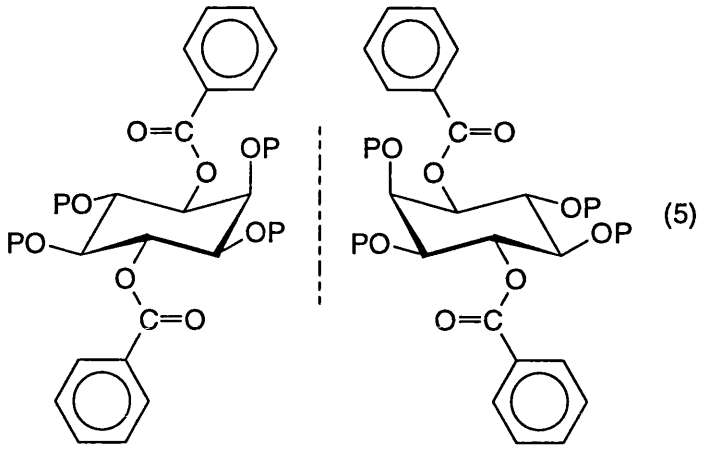
(2)



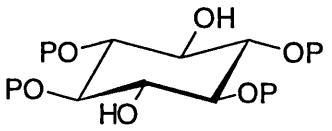
(3)



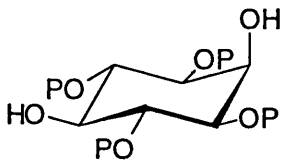
(4)



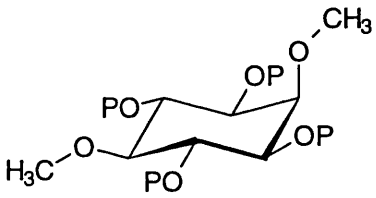
(5)



(6)



(7)



(8)

Figure 4.5. Inositol tetrakisphosphates (1, D/L-*myo*-Ins(1,3,4,5)P_4; 2, *scyllo*-Ins(1,3,4,5)P_4; 3, D/L-*myo*-Ins(1,2,4,6)P_4; 4, D/L-*myo*-Ins(1,2,4,5)P_4; 5, D/L-3,6-di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4; 6, *scyllo*-Ins(1,2,4,5)P_4; 7, *myo*-Ins(1,3,4,6)P_4; 8, 2,5-di-*O*-methyl *myo*-Ins(1,3,4,6)P_4)

The iron uptake profiles associated with this range of inositol tetrakisphosphates are illustrated in Figures 4.6 (p80) and 4.7 (p81) and the data are summarised in Table 4.2.

Inositol Polyphosphate	**Initial Rate of ^{55}Fe Uptake (pmol/min/10^9cells)**	**^{55}Fe Accumulated After 30 min (pmol/10^9 cells) (Mean±SEM, n=3)**
D-*myo*-Ins(1,3,4,5)P_4	10.26	49.14±3.41
L-*myo*-Ins(1,3,4,5)P_4	8.70	47.62±0.70
***scyllo*-Ins(1,3,4,5)P_4**	8.00	43.37±5.42
D/L- *myo*-Ins(1,2,4,6)P_4	3.05	9.76±4.53
D/L *myo*-Ins(1,2,4,5)P_4	25.00	95.83±19.93
D/L di-*O*-bz *myo*-InsP_4	2.00	12.40±5.10
D/L *scyllo*-Ins(1,2,4,5)P_4	24.39	73.49±12.50
***myo*-InsP_6**	1.63	19.08±1.59

Table 4.2. Inositol tetrakisphosphate-mediated iron transport into *P. aeruginosa* PAO1. *myo*-InsP_6-mediated iron transport is duplicated for comparison.

In addition to the compounds in table 4.2, *myo*-Ins(1,3,4,6)P_4 and 2,5 di-*O*-methyl *myo*-Ins(1,3,4,6)P_4 were tested for their ability to mediate iron transport into *P. aeruginosa*. *myo*-Ins(1,3,4,6)P_4, was still capable of chelating iron in this system although insufficient was available to produce an iron transport profile. In contrast, 2,5 di-*O*-methyl *myo*-Ins(1,3,4,6)P_4 was unable to chelate iron in this transport assay and precipitation was always observed.

4.5. Inositol Trisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1.

The final group of inositol phosphates to be tested were the inositol trisphosphates and are illustrated below.

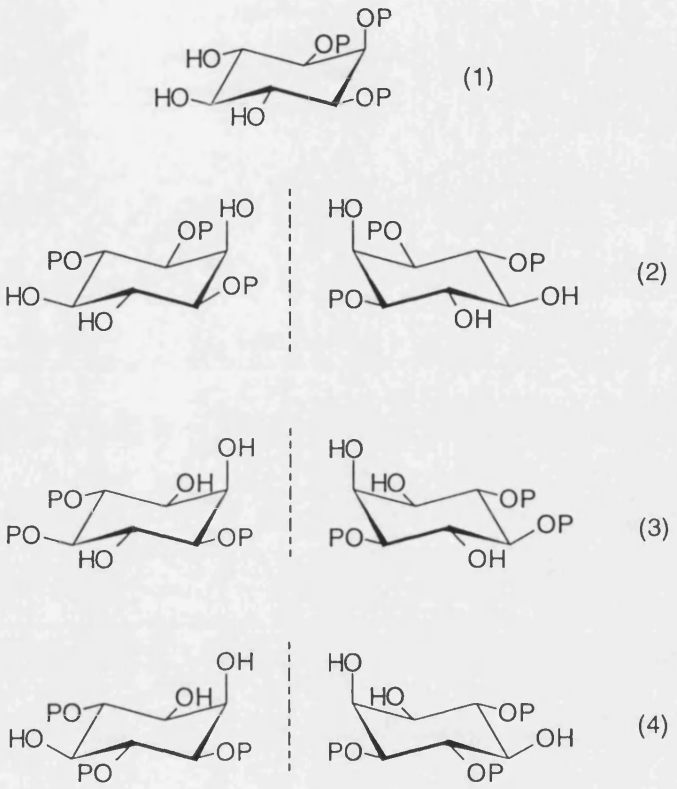


Figure 4.8. myo-Inositol Trisphosphates (1, *myo*-Ins(1,2,3)P_3; 2, D/L-*myo*-Ins(1,3,4)P_3; 3, D/L-*myo*-Ins(1,4,5)P_3; 4, D/L-*myo*-Ins(1,4,6)P_3).

Inositol trisphosphate-mediated iron transport is indicated by figure 4.9. (p82) and the data are summarised below.

Inositol Polyphosphate	Initial Rate of ^{55}Fe Uptake (pmol/min/10^9 cells)	^{55}Fe Accumulated After 30 min (pmol/10^9 cells) (Mean n=3)
myo-Ins(1,2,3)P_3	3.15	11.61±1.39
D/L-*myo*-Ins(1,3,4)P_3	11.32	20.06±2.31
D-*myo*-Ins(1,4,5)P_3	55.56	106.37±6.57
D/L-*myo*-Ins(1,4,6)P_3	41.67	70.30±1.67
myo-InsP_6	1.63	19.08±1.59

Table 4.3. myo-Inositol trisphosphate-mediated iron transport into *P. aeruginosa* PAO1. Transport data for *myo*-InsP_6 are duplicated for comparison.

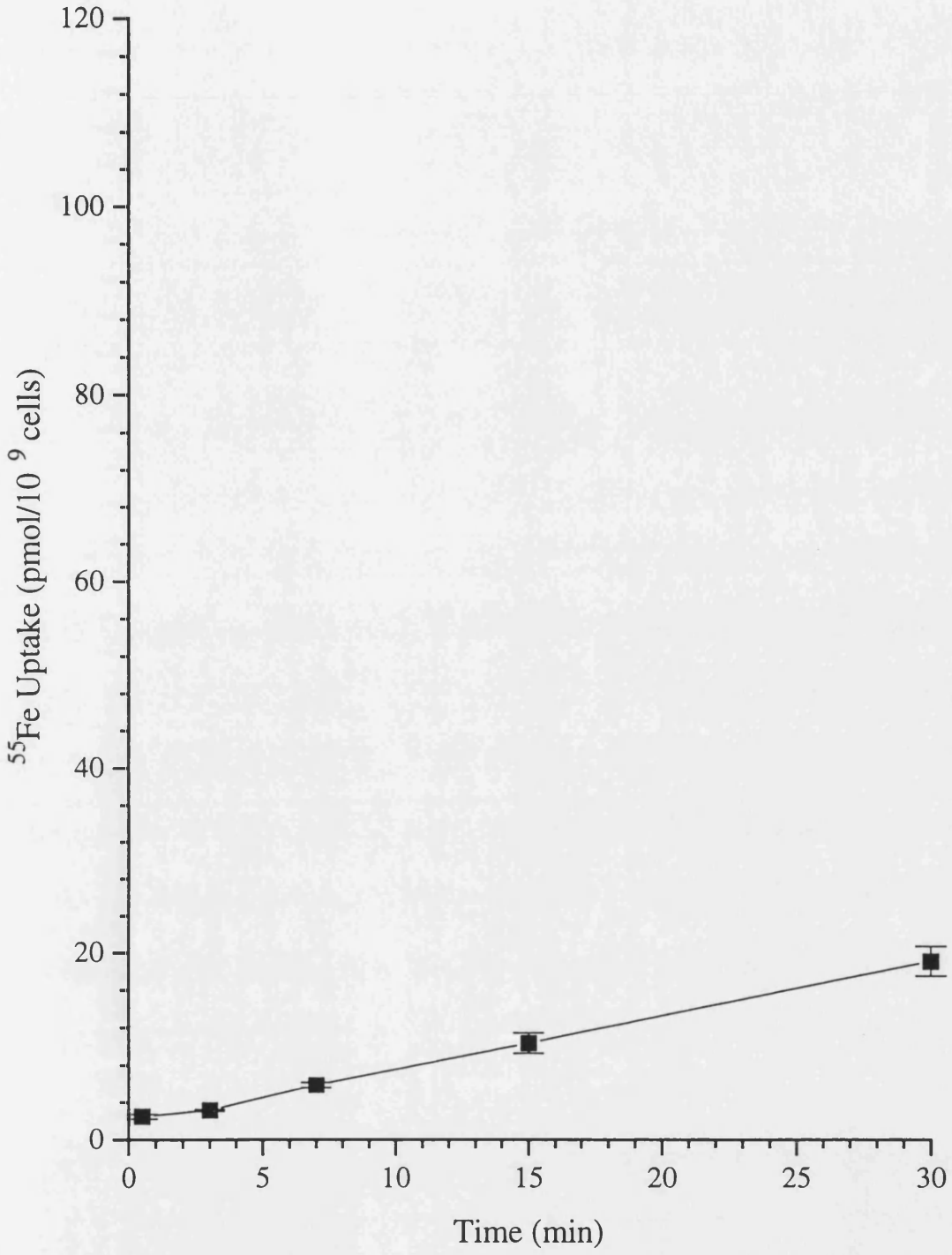


Figure 4.2. *myo*-$InsP_6$-mediated iron transport into *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium contained *myo*-$InsP_6$ (100μM), $^{55}FeCl_3$ (200nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0.

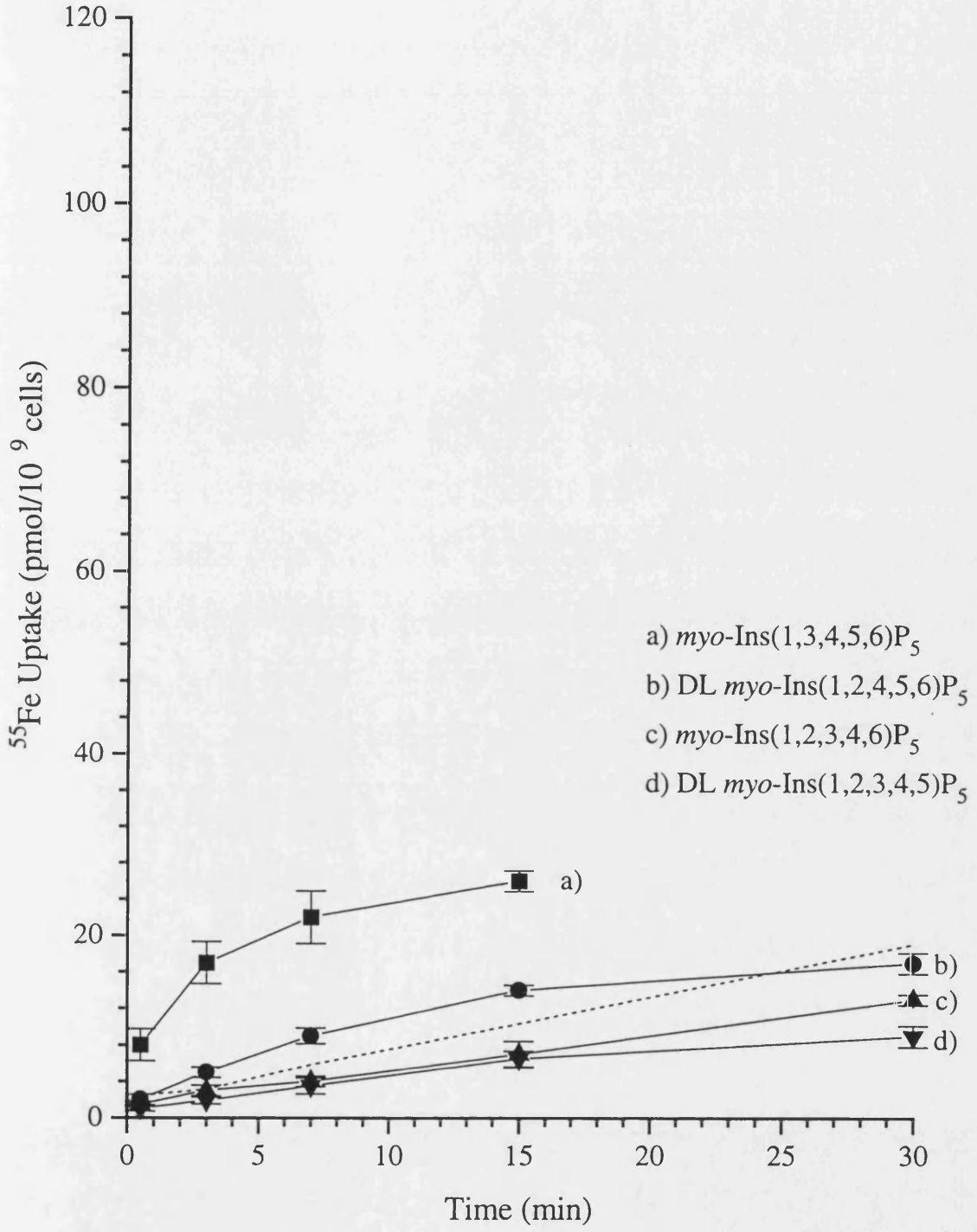


Figure 4.4. Inositol pentakisphosphate-mediated iron transport into *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium contained inositol pentakisphosphate (100 μM), $^{55}FeCl_3$ (200 nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line represents *myo*-InsP_6-mediated transport for comparison.

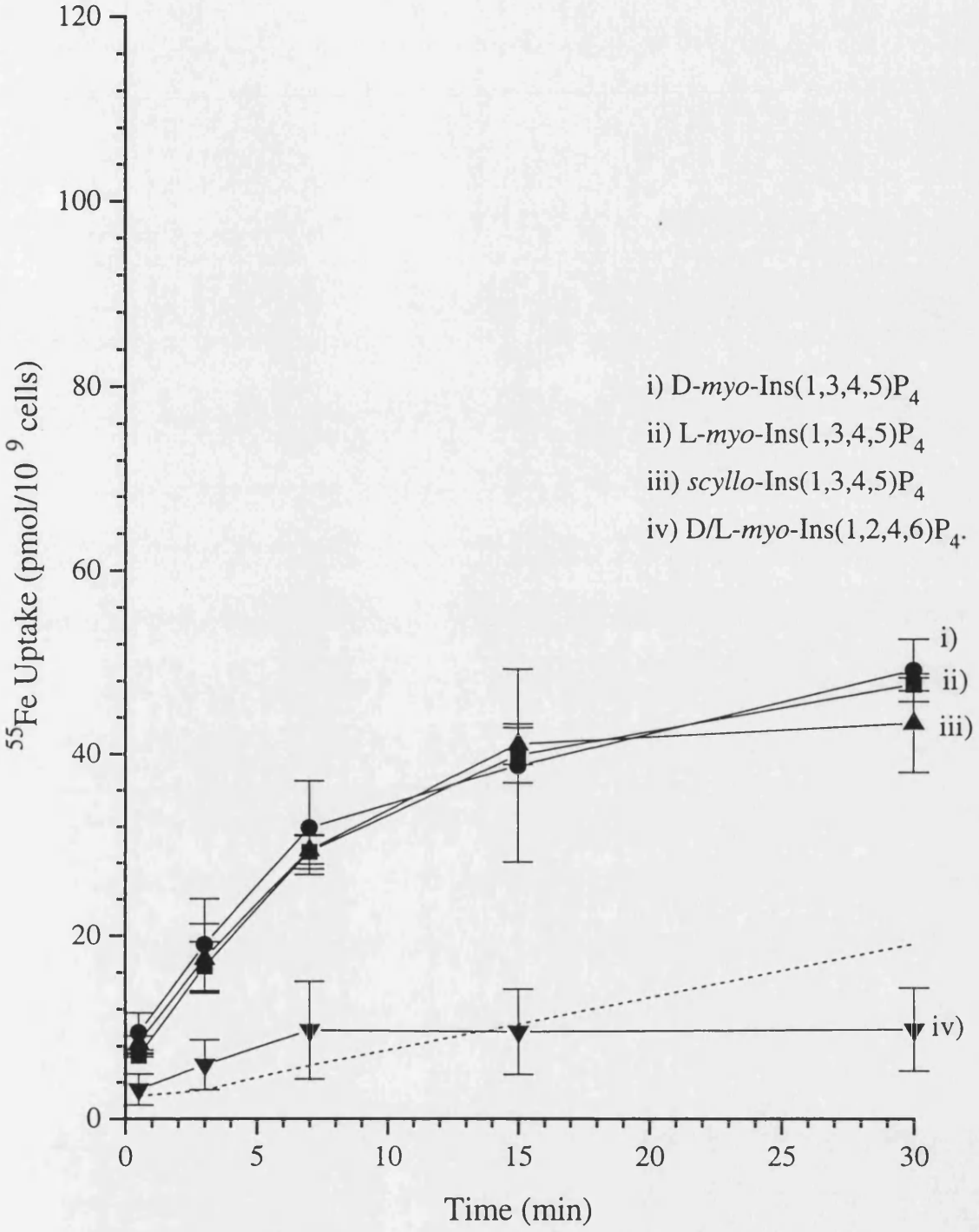


Figure 4.6. Inositol tetrakisphosphate-mediated iron transport into *P. aeruginosa* PAO1 grown in succinate medium. The uptake media contained inositol tetrakisphosphate (100μM), $^{55}FeCl_3$, (200nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line represents *myo*-InsP_6-mediated transport and is duplicated for comparison.

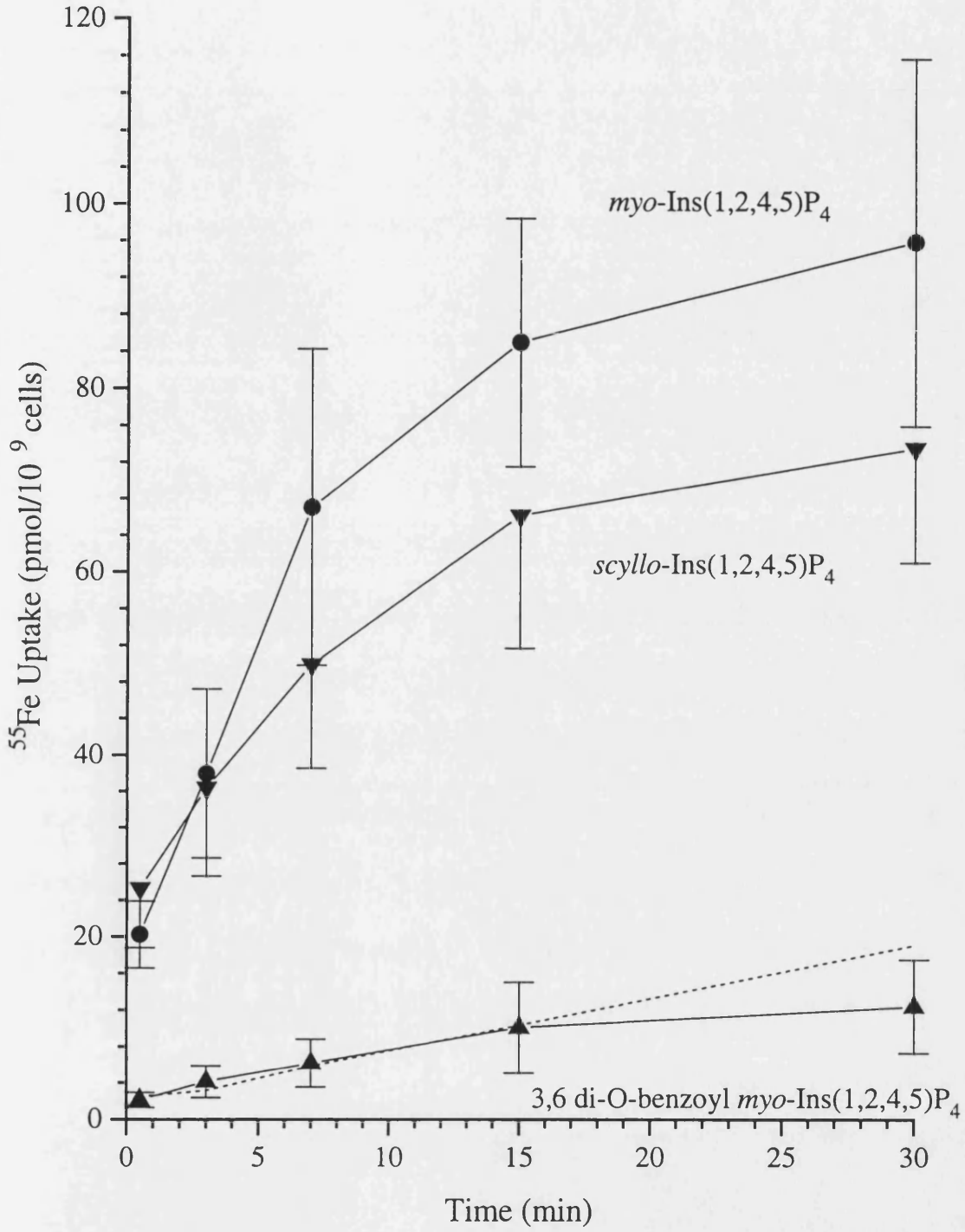


Figure 4.7. Inositol tetrakisphosphate-mediated iron transport into *P. aeruginosa*PAO1 grown in succinate medium. The uptake media contained inositol tetrakisphosphate (100 μM), $^{55}FeCl_3$ (200 nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line represents *myo*-InsP_6-mediated iron transport for comparison.

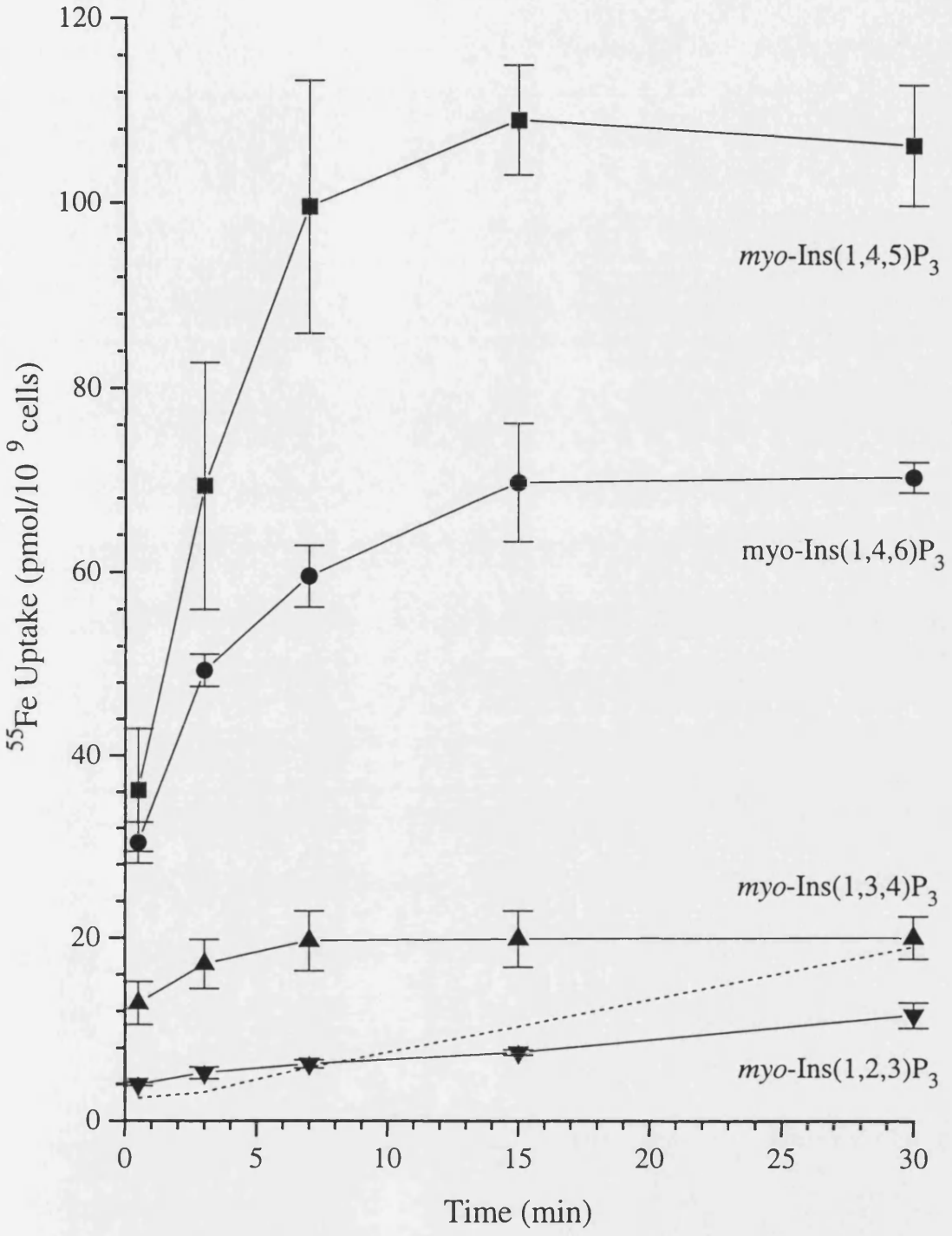


Figure 4.9. Inositol trisphosphate-mediated iron transport into *P. aeruginosa* PAO1 grown in succinate medium. The uptake media contained inositol trisphosphate (100 μM), $^{55}FeCl_3$(200nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line represents *myo*-InsP_6-mediated iron transport for comparison.

4.6. Discussion

Inositol phosphate-mediated iron transport assays provided a basis for the determination of several structure-iron transport relationships. *myo*-$InsP_6$ is a naturally occurring compound that is ubiquitous throughout nature and was used as standard against which the other compounds were compared. It is important to note that the majority of compounds tested are either natural analogues that are not thought to be found in the natural environment of *P. aeruginosa* at levels capable of mediating iron transport or are entirely synthetic entities. These compounds therefore serve to identify key structural motifs and not to mimic environmental conditions.

The Fe(III) chelating ability of *myo*-$InsP_6$ is well documented (with an affinity constant between 10^{25}-10^{30}) (Poyner *et al.*, 1993) and is thought to form the basis of the antioxidant abilities of this compound (Graf *et al.*, 1987). *myo*-$InsP_6$ adopts a chair conformation and has six phosphate groups arranged around its cyclohexane ring. The orientations of the phosphate groups depend on the surrounding environment. When present in solution as the protonated free acid, five of the phosphate groups, in positions 1-, 3-, 4-, 5- and 6-, are arranged equatorially whereas the 2- position phosphate group is in the axial position (5e/1a). However, when present as either the crystalline form or as the dodecasodium salt, a conformational change occurs. This results in the formation of a chair conformation with five phosphate groups (1-, 3-, 4-, 5- and 6-) arranged axially and the remaining phosphate group (2-) in the equatorial position (5a/1e) (Blank *et al.*, 1975; Arnone and Perutz, 1974). ^{31}P NMR studies suggest that the change in conformation of the ring occurs at

pH 9.4 (Isbrandt and Oertel, 1980). The adaptation to the 5a/1e orientation results in each axial ester oxygen becoming in steric opposition with at least one other axial oxygen. Such steric repulsion results in changes in bond angles, torsion angle and intramolecular distances. Varying degrees of stress are also introduced between vicinal phosphate groups. However, vicinal phosphate groups in the 4- and 5-positions are *trans* related hence the steric repulsion between these two groups is minimal. The *cis* arrangement of phosphate groups in the 1-and 2- positions introduces further stress into the molecule hence the 5a/1e orientation is regarded as sterically unfavourable (Blank *et al.*, 1975). Consequently, conversion to the 5e/1a form in aqueous conditions is thought to afford a more sterically favourable orientation. Moreover, Costello *et al.*, (1976) suggested that at high concentrations of $InsP_6$, the conformation of the phytate anion may change to the boat orientation which is considerably more compact and would be favoured as the solvent becomes increasingly depleted.

Iron transport assays were performed using *myo*-$InsP_6$ in dilute aqueous solution (100 μM) hence promoting the 5e/1a conformation. It is this structural arrangement of *myo*-$InsP_6$ that led Hawkins *et al.* (1993) to propose that the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif is important in iron chelation. This motif may, therefore, be an important factor in the ability of *myo*-$InsP_6$ to chelate iron and present it to the bacterium.

Of the six isomers of *myo*-inositol pentakisphosphate, only *myo*-Ins(1,3,4,5,6)P_5 is found at intracellular concentrations similar to those of *myo*-$InsP_6$ (Stephens *et al.*,

1991). *myo*-Ins(1,3,4,5,6)P_5 is the major $InsP_5$ in bovine brain and platelets (Phillippy and Bland, 1988), although in plant extracts such as soya bean, it was present only in low concentrations (Phillippy and Bland, 1988). Although its exact biological role is unclear, *myo*-Ins(1,3,4,5,6)P_5 has been shown to be an intermediate in both the synthesis and degradation of *myo*-$InsP_6$ in mung beans (Biswas *et al.*, 1978). In a number of amphibians and in the majority of birds, *myo*-Ins(1,3,4,5,6)P_5 serves as a modulator of the oxygen affinity of haemoglobin and is thought to function in a manner similar to mammalian 2,3-diphosphoglycerate (Bartet, 1982). In addition, Vallejo *et al.*, (1987) reported that *myo*-Ins(1,3,4,5,6)P_5 had the ability to excite neurons in the brain stems of rats hence having the potential as a neutransmitter.

Because *myo*-Ins(1,3,4,5,6)P_5 is commercially available and is easily extracted from avian blood, it has been tested in more biological assays than any other inositol pentakisphosphates. Consequently, the lack of availability of the other isomers has resulted in a relative lack of knowledge regarding these molecules.

When studying the nomenclature and stereochemistry of inositol pentakisphosphates, it must be remembered that a plane of symmetry exists down the 2/5 axis. Consequently, of the six isomers of *myo*-$InsP_5$, two are meso- compounds, *myo*-Ins(1,3,4,5,6)P_5 and *myo*-Ins(1,2,3,4,6)P_5 whilst *myo*-Ins(1,2,4,5,6)P_5 and *myo*-Ins(1,2,3,4,5)P_5 are present as racaemic pairs as illustrated in figure 4.3. The enantiomeric pairs listed above exist in a 50:50 mixture hence the designations D/L or ± are used. D-*myo*-Ins(2,3,4,5,6)P_5 is not represented because this is the same as

L-*myo*-Ins(1,2,4,5,6)P_5 and similarly, D-*myo*-Ins(1,2,3,5,6)P_5 is the same as L-*myo*-(1,2,3,4,5)P_5.

Iron transport assays using inositol pentakisphosphates indicated that loss of the 2-position phosphate group resulted in enhanced iron transport compared with *myo*-InsP_6. In contrast, loss of the 3-position phosphate represented by D/L *myo*-Ins(1,2,4,5,6)P_5 had little effect on the ability of the compound to mediate iron transport despite a slight increase in the initial rate. Table 4.1. indicates that removal of either the 5- or 6- position phosphate groups had detrimental effects on inositol phosphate-mediated iron transport. The detrimental effect was most noticeable with D/L *myo*-Ins(1,2,3,4,5)P_5 which lacks the 6-position phosphate group.

Interestingly, the two inositol pentakisphosphates retaining the vicinal 1,2,3 (axial, equatorial, axial) trisphosphate motif, thought to be important in iron binding (Hawkins *et al.*, 1993), were less able to mediate iron transport than those lacking this motif. Also of interest is the activity surrounding the 5-position phosphate group. Studies examining the ability of *myo*-Ins(1,4,5)P_3 to act at the *myo*-Ins(1,4,5)P_3 receptor indicated that the 4,5 bisphosphate motif is important in receptor recognition (reviewed by Potter and Lampe 1995). In this study, it also appears that disruption of the 4,5-bisphosphate motif by removal of the 5-phosphate group results in reduced activity. Studies observing structure-activity relationships at the eukaryotic *myo*-Ins(1,4,5)P_3 receptor indicate that changes in functional group at the 6-position are important (Polokoff *et al.*, 1988). This study similarly indicates

that in inositol phosphate-mediated iron transport, changes in functional group at the 6-position results in notably altered activity .

Iron transport assays were also performed using various inositol tetrakisphosphates. Several forms of Ins(1,3,4,5)P_4 were assayed for their abilities to mediate iron transport into *P. aeruginosa* PAO1. All forms of Ins(1,3,4,5)P_4, which do contain the 4,5-bisphosphate motif, were good mediators of iron transport and were considerably greater better than *myo*-InsP_6.

myo-Ins(1,3,4,5)P_4 is a naturally occurring compound although it is not thought to exist in the natural environment of *P. aeruginosa.* It is a metabolite of *myo*-Ins(1,4,5)P_3 produced as a result of 3-kinase activity. The exact role of *myo*-Ins(1,3,4,5)P_4 in eukaryotic systems is unclear although it does appear to have modest calcium mobilising activity (Cullen *et al.,* 1994). The role of 3-kinase is particularly important and was initially thought to provide a mechanism of terminating the response elicited by *myo*-Ins(1,4,5)P_3 by virtue of *myo*-Ins(1,3,4,5)P_4 having little physiological effect. However, more recently, specific binding sites for this compound have been found located in the plasma membrane of human platelets suggesting a more distinct role in cellular signalling. This is in contrast to the *myo*-Ins(1,4,5)P_3 receptors which are normally found associated with intracellular membranes (Cullen *et al.*, 1994).

It was particularly interesting to note that both D- and L-*myo*-Ins(1,3,4,5)P_4 produced near identical iron uptake profiles as indicated in figure 4.6. In mammalian studies

of *myo*-Ins(1,4,5)P_3 acting at the Ins(1,4,5)P_3 receptor, strong stereospecificity was observed. L-*myo*-Ins(1,4,5)P_3 was unable to mobilise calcium and binding of this enantiomer was approximately 2,000-fold weaker than that of the natural enantiomer, D- *myo*-Ins(1,4,5)P_3 (Strupish *et al.*, 1988). This stereospecificity was also observed with several *myo*-Ins(1,4,5)P_3 analogues acting as substrates for 3-kinase and 5-phosphatase (Safrany *et al.*, 1992). The apparent lack of specificity of D- and L-*myo*-Ins(1,3,4,5)P_4 suggests that inositol phosphate mediated iron transport does not occur *via* specific inositol phosphate receptors.

Studies using *scyllo*-Ins(1,3,4,5)P_4, where the 2-position hydroxyl group is in the equatorial rather than the axial orientation, produced an iron uptake profile that was near identical to the *myo*- derivatives. Previous studies examining the activity of *myo*-Ins(1,3,4,5)P_3 at the *myo*-Ins(1,4,5)P_3 receptor suggest that changes of conformation at the 2-position were also well tolerated (Wilcox *et al.*, 1993).

The synthetic compound D/L-*myo*-Ins(1,2,4,6)P_4 was also tested. This compound was of particular interest because of the three vicinal equatorial phosphate groups in the 1-, 2- and 6-positions. In common with compounds possessing the 1,2,3 (equatorial, axial, equatorial) motif, this compound was also a poor mediator of iron transport.

Several Ins(1,2,4,5)P_4 derivatives were tested. *myo*-Ins(1,2,4,5)P_4 is a synthetic compound designed to determine the effects of substituting the 2-position hydroxyl group of *myo*-Ins(1,4,5)P_3 with another phosphate group. *myo*-Ins(1,2,4,5)P_4 had

considerable calcium mobilising activity in eukaryotic systems (Mills *et al.*, 1993b) and was found to function as a full agonist with only 3-fold less potency than D-*myo*-Ins(1,4,5)P_3. In addition *myo*-Ins(1,2,4,5)P_4 was resistant to both Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase (Hirata *et al.*, 1994).

In this study, *myo*-Ins(1,2,4,5)P_4 was a particularly effective mediator of iron uptake into *P. aeruginosa.* Interestingly, this compound retains the 4,5-bisphosphate motif which, in eukaryotic studies, is considered vital in calcium mobilisation (Berridge and Irvine, 1984). A preliminary study using D- and L-enantiomers of *myo*-Ins(1,2,4,5)P_4 produced near identical results which was consistent with data from with D- and L-*myo*-Ins(1,3,4,5)P_4 (data not shown).

Additional transport assays were performed using a sterically modified version of *myo*-Ins(1,2,4,5)P_4, produced by addition of *O*-benzoyl groups at the 3- and 6-positions. 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 has the same arrangement of phosphate groups although substitutes two hydroxyl groups for -*O*-benzoyl groups. This compound was assayed in order to determine whether addition of large functional groups afforded any variation in the ability to mediate iron transport into *P. aeruginosa.* The resulting iron transport associated with this compound was very much less than that observed with *myo*-Ins(1,2,4,5)P_4, and was similar to that achieved using *myo*-InsP_6. Of two possible explanations, one may be that substitution of hydroxyl groups in the 3- and 6- positions with less charged *O*-benzoyl groups, reduces the ability of this compound to complex iron and therefore deliver it back to the bacterium. Alternatively, addition of large, sterically hindering

groups, may reduce the ability of the compound to come into close contact with the surface of the bacterium hence reducing the ability of the compound to yield iron to a putative transport system.

Iron transport assays were also performed using *scyllo*-Ins(1,2,4,5)P_4 which differs from *myo*-Ins(1,2,4,5)P_4 with respect to the orientation of the 2-position phosphate group. *scyllo*-Ins(1,2,4,5)P_4-mediated iron transport was similar to that achieved using *myo*-Ins(1,2,4,5)P_4 which is consistant with D- and L-*myo*- and *scyllo*-Ins(1,3,4,5)P_4 where the change in orientation at the 2-position was of little consequence.

It was unfortunate that *myo*-Ins(1,3,4,6)P_4 was not available in sufficient quantities to perform transport assays However, this compound was able to chelate iron as judged by the relatively low counts on the nitrocellulose filters. Interestingly, 2,5 di-*O*-methyl *myo*-Ins(1,3,4,6)P_4 was unable to chelate iron in this assay which may be a result of the substitution of the charged hydroxyl groups with less charged -*O*-methyl groups. This suggests that two vicinal phosphate groups are not sufficient to chelate iron without the presence of at least one vicinal hydroxide group. The ability of 3,6-di-*O*-benzoyl-*myo*-Ins(1,2,4,5)P_4 to chelate iron may be associated with the charge surrounding the carbonyl group of the -*O*-benzoyl configuration.

The final group of compounds tested were the inositol trisphosphates. Figure 4.9. illustrates that these compounds produced a diverse range of iron transport profiles ranging from *myo*-Ins(1,2,3)P_3, which produced a final accumulated amount of iron

of 11.61±1.39 pmol/10^9 cells, to *myo*-Ins(1,4,5)P_3, which produced a final accumulated amount of iron of 106.37±6.57 pmol/10^9 cells. Interestingly, the 1,2,3 (axial, equatorial, axial) trisphosphate motif of *myo*-Ins(1,2,3)P_3 is considered important for iron binding (Hawkins *et al.*, 1993) whereas the 4,5 bisphosphate motif of *myo*-Ins(1,4,5)P_3 appears important in calcium release in eukaryotic studies.

myo-Ins(1,2,3)P_3 provides the minimal structure required for assessing the effect of the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif. *myo*-Ins(1,2,3)P_3 is a naturally occurring inositol trisphosphate that has been located in numerous eukaryotic cells in the concentration range 1-10 μM (Barker *et al.*, 1995). It is thought that *myo*-Ins(1,2,3)P_3 is produced as a result of "phytase"-mediated dephosphorylation of *myo*-InsP_6 (Cosgrove, 1969) and is unusual in that the majority of naturally occurring inositol phosphates do not possess a 2-position phosphate group (Barker *et al.*, 1995). A study by Hawkins *et al.*, (1993) suggested that compounds possessing the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif, which includes both *myo*-Ins(1,2,3)P_3 and *myo*-InsP_6, are particularly efficient at chelating Fe(III). Coupled with the observations that the cellular turnovers of both *myo*-Ins(1,2,3)P_3 and *myo*-InsP_6 are extremely slow, these findings suggest that the physiological role of *myo*-Ins(1,2,3)P_3 may be linked to intracellular antioxidant properties (Barker *et al.*, 1995). Iron transport assays using this compound strengthened the observation that the presence of this motif results in poor iron transport which was almost half that achieved using *myo*-InsP_6. Unlike *myo*-InsP_6, this compound does not posses the vicinal 4,5 bisphosphate motif which appears important for the iron uptake mediating activity of these compounds.

The eukaryotic second messenger *myo*-Ins(1,4,5)P_3 was the best mediator of iron transport tested in this study. The first reports of the second messenger activity were in 1983 and 1984 (Streb *et al.*, 1983; Berridge and Irvine, 1984) when it was realised that this molecule provided the missing link between receptor stimulation and mobilisation of calcium from intracellular stores. This discovery has provided understanding of many receptor-coupled aspects of cellular control (Potter, 1992). Interestingly, this compound retains the 4,5 bisphosphate motif considered essential for the ability of this compound to act at the eukaryotic *myo*-Ins(1,4,5)P_3 receptor. It also highlights the relative importance of alterations at the 2-position when compared to *myo*-Ins(1,2,4,5)P_4 as noted previously. In comparison to *myo*-Ins(1,2,4,5)P_4, it illustrates that a change of functional group at the 2-position has some effect on the ability to mediate iron transport into *P. aeruginosa*. These results mirror those of the eukaryotic calcium mobilisation studies by Mills *et al.*, (1993b). In common with *myo*-Ins(1,3,4,5)P_4 and *myo*-Ins(1,2,4,5)P_4, a preliminary study illustrated no difference between the activity of the D- and L- enantiomers of *myo*-Ins(1,4,5)P_3 suggesting further, the lack of a specific receptor (data not shown).

Iron transport assays were also performed using *myo*-Ins(1,3,4)P_3. This compound is a naturally occurring inositol phosphate produced by the sequential action of Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase. In mammalian cells, the signal generated by *myo*-Ins(1,4,5)P_3 can be terminated by 5-phosphatase to yield D-*myo*-Ins(1,4)P_2, and also by 3-kinase to produce D-*myo*-Ins(1,3,4,5)P_4. In addition to the direct metabolism of Ins(1,4,5)P_3, 5-phosphatase is also capable of removing the 5-

phosphate group from Ins(1,3,4,5)P_4 to yield Ins(1,3,4)P_3 (Berridge and Irvine 1989; van Dijken *et al.*, 1994). In soluble extracts of porcine skeletal muscle Ins(1,3,4)P_3 is subsequently dephosphorylated to D-*myo*-Ins(3,4)P_2, D-*myo*-Ins(3)P and finally to free inositol (Foster *et al.*, 1994). Alternatively, a 5/6 kinase has been demonstrated to act upon *myo*-Ins(1,3,4)P_3 to produce *myo*-Ins(1,3,4,6)P_4 and *myo*-Ins(1,3,4,5)P_4 (Hughes *et al.*, 1994) with a 6-kinase:5-kinase activity ratio of 4:1.

myo-Ins(1,3,4)P_3 was a relatively poor mediator of iron transport and gave a profile only slightly greater than that achieved using *myo*-InsP_6. This observation again suggests that the 5-position phosphate group appears important in the ability to mediate iron transport with disruption of the 4,5-bisphosphate motif resulting in reduced iron transport.

The final inositol trisphosphate to be tested was *myo*-Ins(1,4,6)P_3. *myo*-Ins(1,4,6)P_3 is a synthetic compound, originally designed to determine structure-activity relationships of *myo*-Ins(1,4,5)P_3 and the relative importance of the 4,5-bisphosphate motif in mediating intracellular Ca^{2+} release (Mills *et al.*, 1993a). In eukaryotic systems, it was noted that the naturally occurring inositol tetrakisphosphate *myo*-Ins(1,3,4,6)P_4 possessed Ca^{2+} mobilising activity despite the absence of the 4,5-bisphosphate motif (Polokoff *et al.*, 1988 and Ivorra *et al.*, 1991). The subsequent synthesis of *myo*-Ins(1,4,6)P_3 revealed that this compound had 11-fold less potent calcium mobilising activity than *myo*-Ins(1,4,5)P_3 but was 5-fold more potent than the natural compound, *myo*-Ins(1,3,4,6)P_4 (Mills *et al.*, 1993a). These data led Mills

et al., (1993a) to suggest that the 1,6-bisphosphate configuration mimics the normal 4,5-bisphosphate motif in *myo*-Ins(1,4,5)P_3.

A similar phenomenon was observed with *myo*-Ins(1,4,6)P_3-mediated iron transport whereby the iron transport activity associated with this compound was greater than expected when considering the lack of the 4,5-bisphosphate motif. This gives further suggestion that the 1,6-bisphosphate motif may mimic the normal 4,5-bisphosphate motif in *myo*-Ins(1,4,5)P_3.

In summary, this chapter has identified two key structural motifs involved in inositol phosphate-mediated iron transport into *P. aeruginosa.* The presence of the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif appears to be associated with a low iron uptake profile. This motif has been suggested to play a key role in high affinity iron chelation (Hawkins *et al.*, 1993) and chapter 5 will attempt to determine whether the low iron transport associated with this motif is a result of iron being withheld from a putative transport system. Also, the addition of large sterically hindering groups reduces iron transport which may represent a reduced interaction between the ferri-inositol phopshate complex and a putative carrier system. The other key group is the 4,5-bisphosphate motif. Studies using the lower inositol phosphates suggest that compounds retaining this arrangement are better mediators of iron transport. This is in common wth eukaryotic calcium mobilisation studies whereby the presence of the 4,5-bisphosphate group is considered essential (Berridge and Irvine, 1984).

This chapter also illustrates that changes at the 2- and 3-positions have modest effects on iron transport. In comparison with *myo*-Ins(1,4,5)P_3, addition of a phosphate group to the 3-position (*myo*-Ins(1,3,4,5)P_4) was more detrimental to iron transport than the addition of a phosphate group to the 2-position (*myo*-Ins(1,2,4,5)P_4). These data mirror those of eukaryotic studies in that *myo*-Ins(1,3,4,5)P_4 had minimal calcium mobilising activity whereas myo-Ins(1,2,4,5)P_4 was considerably more potent. Using *scyllo*- and *myo*-derivatives of Ins(1,2,4,5)P_4 and Ins(1,3,4,5)P_4, it was apparent that orientation of the 2-position functional groups was of minor importance. Also of importance were the observations that both the D- and L- forms of Ins(1,3,4,5)P_4, Ins(1,2,4,5)P_4 and Ins(1,4,5)P_3 mediated similar iron uptake suggesting the lack of a specific receptor.

Chapter 5

The Ability of Inositol Phosphates to Interact With Ferric Iron Relative to the Ability to Mediate Iron Transport in *Pseudomonas aeruginosa*

5.1 Introduction

Chapter 4 illustrated the ability of several inositol polyphosphates to mediate iron transport into *P. aeruginosa* PAO1 and it was possible to determine various structure-iron transport relationships. Whilst iron transport assays illustrated the ability of the individual inositol polyphosphates to chelate Fe(III), they give no information regarding the relative affinities of each of the compounds towards Fe(III).

Consequently, a series of experiments was performed to gain an insight into the relative abilities of several inositol phosphates to complex Fe(III). A hydroxyl radical generation assay was used to assess the ability of these compounds to bind Fe(III) in a manner that inhibits iron-catalysed hydroxyl free radical generation. Secondly, experiments were performed to determine the uptake profile using a combination of inositol polyphosphates which appear to have different iron complexation abilities. Finally, iron transport assays were performed to assess whether *myo*-$InsP_6$-mediated iron transport confers a competitive advantage in the

environment. This assessed the ability of *myo*-$InsP_6$ to mediate iron transport at different pH values and by measuring the ability of *myo*-$InsP_6$ to compete with a non-utilisable *P. aeruginosa* pyoverdine.

5.2 Hydroxyl Radical Assay

Graf *et al.*, (1987) described the hypoxanthine-xanthine oxidase-based hydroxyl assay to demonstrate the anti-oxidant properties of $InsP_6$, which was later extended to include other inositol polyphosphates by Hawkins *et al.*, (1993). The antioxidant ability results from a complex mechanism in which the chelating ability plays an important role.

Briefly, oxidation of hypoxanthine by xanthine oxidase produces a mixture of hydrogen peroxide, hydroxyl radical and the superoxide anion (Klein *et al.*, 1981). Hydroxyl radicals are produced as a result of the reaction between the superoxide anion and hydrogen peroxide in an iron-catalysed Haber-Weiss type reaction as illustrated below.

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\bullet$$

The interaction of hydroxyl radicals with dimethyl sulphoxide results in the production of methyl radicals which can react in three ways (Klein *et al.*, 1981). Methyl radicals may abstract a hydrogen to produce methane, dimerise to ethane or react with molecular oxygen to give the methylperoxy radical (Me-O-O$^{\cdot}$).

Subsequent decomposition of Me-O-O$^{\cdot}$ can result in the production of formaldehyde which is followed by measurement of absorbance at 410 nm.

$$2\text{Me-O-O}^{\cdot} \rightarrow H_2C{=}O + \text{MeOH} + O_2$$

Consequently, addition of iron-chelating compounds, that render the iron incapable of catalysing the Haber-Weiss reaction, results in reduced hydroxyl radical formation and a subsequent reduction in formaldehyde production (Graf *et al.*, 1984).

In order to catalyse hydroxyl radical generation, iron must have one or more free co-ordination sites or have co-ordination sites occupied by a readily dissociable ligand such as water. The majority of iron chelators, such as EDTA, bind iron in such a manner that retains an active iron co-ordination site. This results in the formation of a complex that will still support hydroxyl radical generation. However, *myo*-$InsP_6$ forms a water-excluding chelate that will not support hydroxyl radical generation (Graf *et al.*, 1987). This effect is observed even at high iron:*myo*-$InsP_6$ ratios and is thought to be due to *myo*-$InsP_6$ shifting the redox potential of iron and moderating the oxidation of Fe(II). Fe(II) can be oxidised either by oxygen, which produces low levels of hydroxyl radicals, or by hydrogen peroxide, which produces high levels of damaging hydroxyl radicals. *myo*-$InsP_6$ accelerates oxygen-mediated oxidation whilst not affecting hydrogen peroxide-mediated oxidation. This results in the conversion of Fe(II) to the relatively inert Fe(III) *via* a low hydroxyl radical generating mechanism which is then chelated in a non-reactive form by *myo*-$InsP_6$. It is these mechanisms which are proposed to give *myo*-$InsP_6$ antioxidant properties *in vivo* (Graf *et al.*, 1987).

Using this method, it was possible to determine the relative abilities of several *myo*-inositol phosphates to inhibit formaldehyde production resulting from iron-catalysed hydroxyl radical generation.

5.2.1. Hydroxyl Assay Results

The abilities of several inositol polyphosphates to inhibit Fe(III)-catalysed hydroxyl radical generation are summarised below.

Inositol Phosphate	% of Control Colour Production (Mean±SEM, n=3)
myo-InsP_6	0
myo-Ins(1,4,6)P_3	7±1
myo-Ins(1,2,3)P_3	31±0.2
myo-Ins(1,2,4,5)P_4	66±3
3,6 di-*O*-benzoic *myo*-Ins(1,2,4,5)P_4	68±4
myo-Ins(1,3,4)P_3	82±4
myo-Ins(1,4,5)P_3	104±1

Table 5.1. Inhibition of hydroxyl radical-mediated formaldehyde production by *myo*-inositol phosphates (100 μM). The values represent the amount of formaldehyde produced, as percentages of control incubations without added inositol phosphates.

5.3. Competition Between Efficient and Poor Mediators of Iron Transport

Data from section 5.2. identified variations in the ability of several inositol phosphates to interact with ferric iron in a manner that prevents iron-catalysed

hydroxyl radical formation. Despite apparent differences in their abilities to interact with iron, these data do not indicate whether these differences are of significant magnitude to allow competition for iron between individual compounds. In order to address this question, iron transport assays were performed containing equimolar (100 μM) concentrations of two inositol phosphate compounds that produced markedly different iron-uptake profiles. The aim of these experiments was to determine whether the inositol phosphate with the greater ability to interact with iron was able to compete for iron and confer an iron-uptake profile normally associated with that compound alone.

5.3.1. Iron Transport Assay Using Equimolar Inositol (1,2,3) Trisphosphate and *myo*-Inositol (1,4,5) Trisphosphate

Iron transport assays were performed using a combination of equimolar (100 μM) *myo*-Ins(1,2,3)P_3 and *myo*-Ins(1,4,5)P_3. From previous data, it was observed that *myo*-Ins(1,2,3)P_3 was able to interact with iron in a manner which inhibited hydroxyl radical formation yet was a poor mediator of iron transport. Conversely, *myo*-Ins(1,4,5)P_3 appeared less able to interact with iron Fe(III) yet was associated with particularly high iron transport. Figure 5.1. (p104) indicates that in the presence of both of these chelators, iron transport is considerably lower than that achieved using *myo*-Ins(1,4,5)P_3 alone although not quite as low as that achieved with *myo*-Ins(1,2,3)P_3 alone.

5.3.2. Iron Transport Assay Using Equimolar *myo*-Inositol Hexakisphosphate and *myo*-Inositol (1,4,5) Trisphosphate.

This uptake assay was also based on competition of equimolar (100 μM) concentrations of *myo*-$InsP_6$ and *myo*-$Ins(1,4,5)P_3$. *myo*-$InsP_6$ was used since this was the only compound in this study capable of complete inhibition of hydroxyl radical generation. Figure 5.2. (p105) illustrates that in an uptake medium containing both of these inositol phosphates, the uptake pattern resembles that of *myo*-$InsP_6$ rather than *myo*-$Ins(1,4,5)P_3$. The ability of *myo*-$InsP_6$ to compete with *myo*-$Ins(1,4,5)P_3$ was notably greater than *myo*-$Ins(1,2,3)P_3$.

5.4 Assessing the Ability of *myo*-Inositol Hexakisphosphate to Confer a Competitive Advantage in the Environment.

This study has provided an insight into structure-activity relationships of various inositol phosphates. However, with the exception of *myo*-$InsP_6$, all the other compounds tested are unlikely to be found in the natural environment of *P. aeruginosa* at levels capable of mediating iron uptake. Consequently, additional iron transport assays were performed to assess whether the siderophore activity of *myo*-$InsP_6$ gave *P. aeruginosa* a competitive advantage in the environment.

5.4.1. Competition of *myo*-$InsP_6$ with *Pseudomonas aeruginosa* ST:06 Pyoverdine

The ability of *P. aeruginosa* to use exogenous siderophores for iron acquisition is well documented (section 1.7) where it is thought it may confer a competitive advantage. In addition to the iron transporting compounds that are utilisible by *P.*

aeruginosa PAO1, this bacterium may also be surrounded by siderophores from other bacteria that cannot be used. One such example of this is the pyoverdine produced by *P. aeruginosa* ST:06 (Pvd:06) which will bind iron, but will not mediate iron transport into *P. aeruginosa* PAO1 (Gensberg, 1994). Iron transport assays were performed in order to determine whether *myo*-$InsP_6$ has sufficient iron binding ability to remove iron from Pvd:06 and mediate iron uptake.

Uptake experiments were performed using a mixture of Pvd:06 (200μM) and *myo*-$InsP_6$ (100μM) and are illustrated by figure 5.3 (p106). It was observed that Pvd:06 alone, does not mediate iron transport into *P. aeruginosa* PAO1 despite its ability to chelate ferric iron. The combination of Pvd:06 and *myo*-$InsP_6$ results in an uptake profile similar to that of Pvd:06.

5.4.2. The Effect of pH on *myo*-Inositol Hexakisphosphate-Mediated Iron-Transport

All the previous iron transport studies were performed at pH 7.0 although *P. aeruginosa* is found in a range of environments of varying pH. This is particularly true for soil environments. Iron transport assays were performed to determine *myo*-$InsP_6$-mediated iron transport at pH 6.0, 6.5, 7.5 and 8.0. The iron transport profiles are illustrated in figure 5.4 (p107) and the data are summarised in table 5.2.

	Initial Rate of ^{55}Fe Uptake (pmol/min/10^9 cells)	^{55}Fe Accumulated After 30 min (pmol/10^9 cells) (Mean±SEM, n=3)
***myo*-InsP$_6$ at pH 6.0**	2.69	22.75±6.64
***myo*-InsP$_6$ at pH 6.5**	1.61	23.87±6.75
***myo*-InsP$_6$ at pH 7.0**	1.63	19.08±1.59
***myo*-InsP$_6$ at pH 7.5**	0.94	10.17±1.64
***myo*-InsP$_6$ at pH 8.0**	0.72	8.91±2.31

Table 5.2. *myo*-InsP$_6$-mediated iron transport into *P. aeruginosa* PAO1 at a range of different pH values. The data at pH 7.0 are duplicated for comparison.

5.4.3. The Effect of pH on the Competition of *myo*-InsP$_6$ with *Pseudomonas aeruginosa* ST:06 Pyoverdine

Data from section 5.4.2 indicate that *myo*-InsP$_6$-mediated iron-transport was enhanced at low pH hence further experiments were performed to determine whether lowering the pH had any effect on the ability of *myo*-InsP$_6$ to compete with Pvd:06 for iron. To examine the effect of low pH, iron transport assays were performed at pH 6.0. The resulting iron transport profile is illustrated in figure 5.5. (p108) where it is apparent that even at pH 6.0, *myo*-InsP$_6$ is still unable to compete with Pvd:06 for iron.

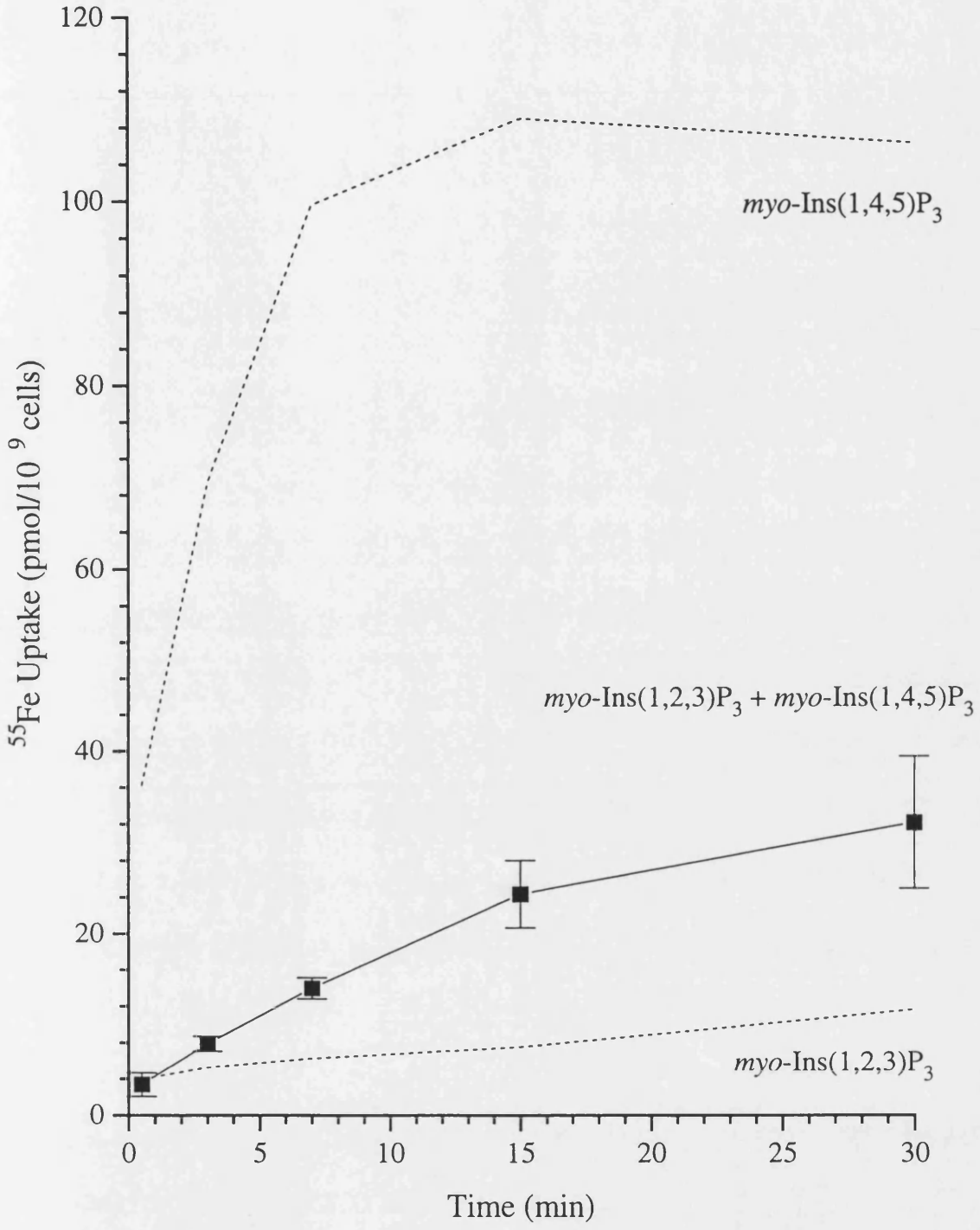


Figure 5.1. Equimolar *myo*-Ins(1,2,3)P_3 and *myo*-Ins(1,4,5)P_3-mediated iron transport in *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium contained *myo*-Ins(1,2,3)P_3 (100μM), *myo*-Ins(1,4,5)P_3 (100μM), $^{55}FeCl_3$ (200nM), glucose (60μM), and 1ml of cells OD_{470} 1.0. The dashed lines represent the compounds individually for comparison.

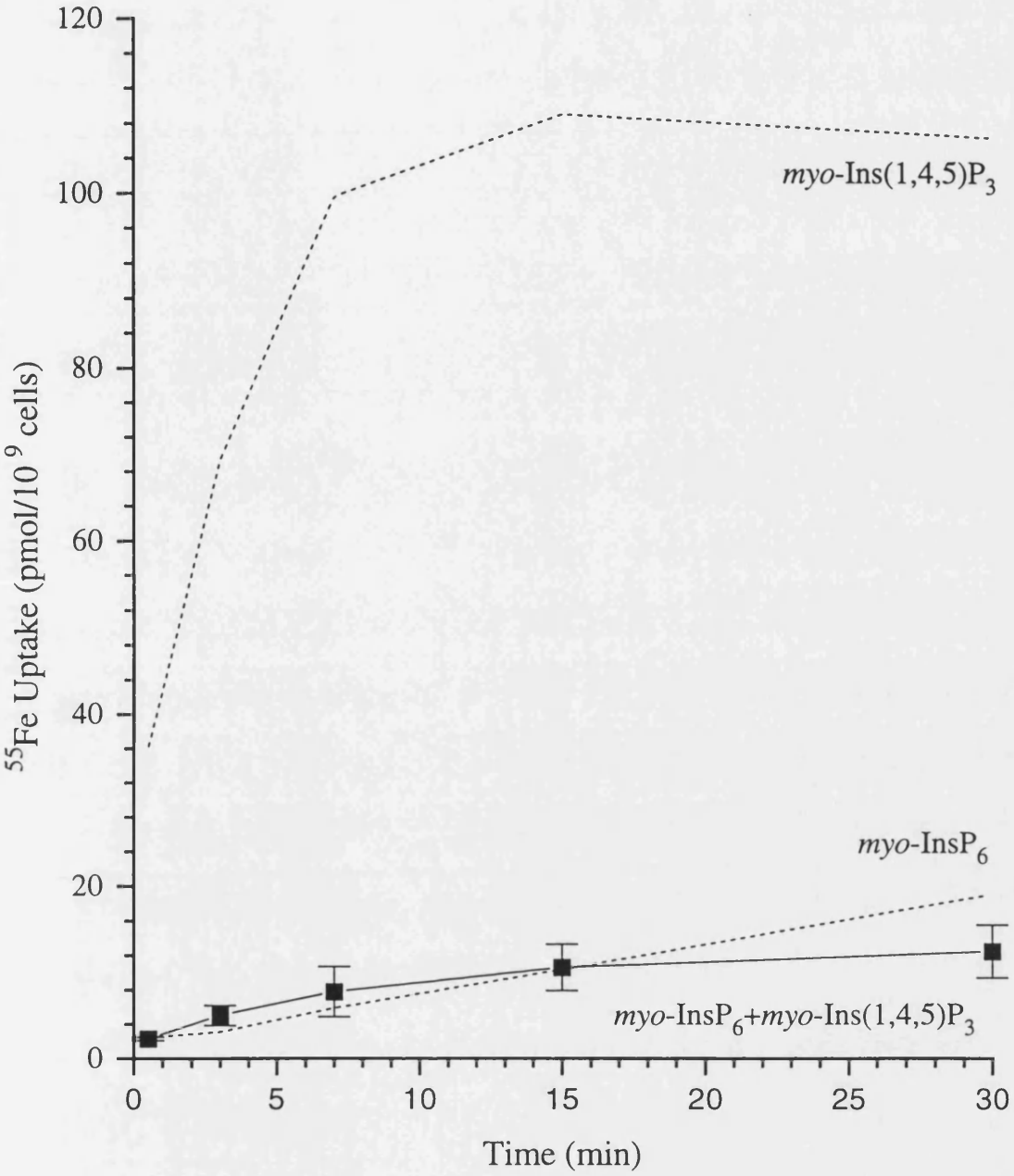


Figure 5.2. Equimolar *myo*-InsP_6 and *myo*-Ins(1,4,5)P_3-mediated iron transport in *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium contained *myo*-InsP_6 (100μM), *myo*-Ins(1,4,5)P_3 (100μM),$^{55}FeCl_3$ (200nM) and 1 ml of cells at OD_{470} 1.0. The dashed line represents normal iron transport associated with *myo*-InsP_6 and *myo*-Ins(1,4,5,)P_3 individually.

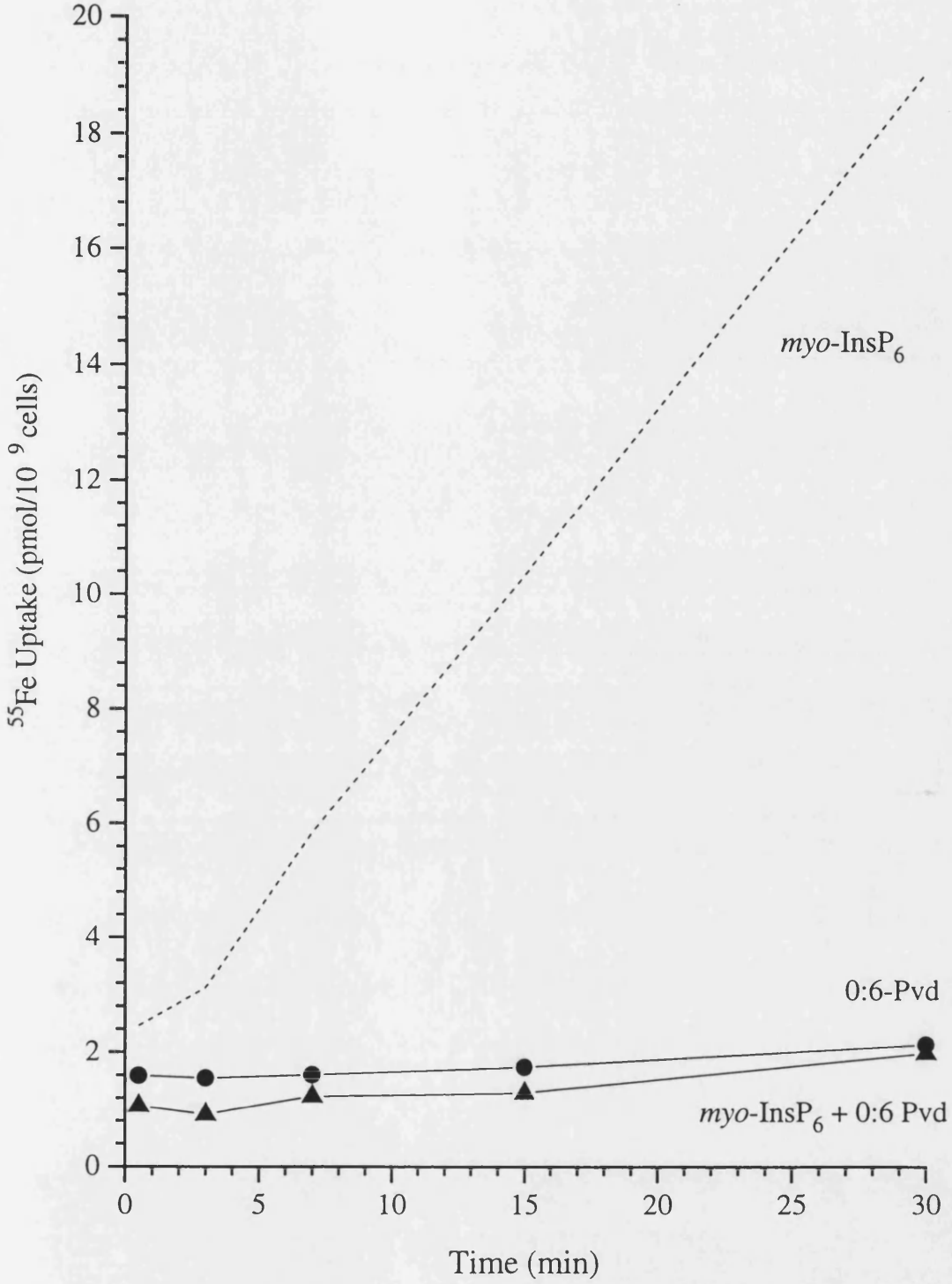


Figure 5.3. The combination of *myo*-InsP$_6$ and O6:Pvd-mediated iron transport in *P. aeruginosa* PAO1 grown in succinate medium. The uptake media contained *myo*-InsP$_6$ (100μM), 0:6 Pvd (200μM), $^{55}FeCl_3$ (200nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line is duplicated from Fig 4.2. for comparison and error bars have been omitted for clarity.

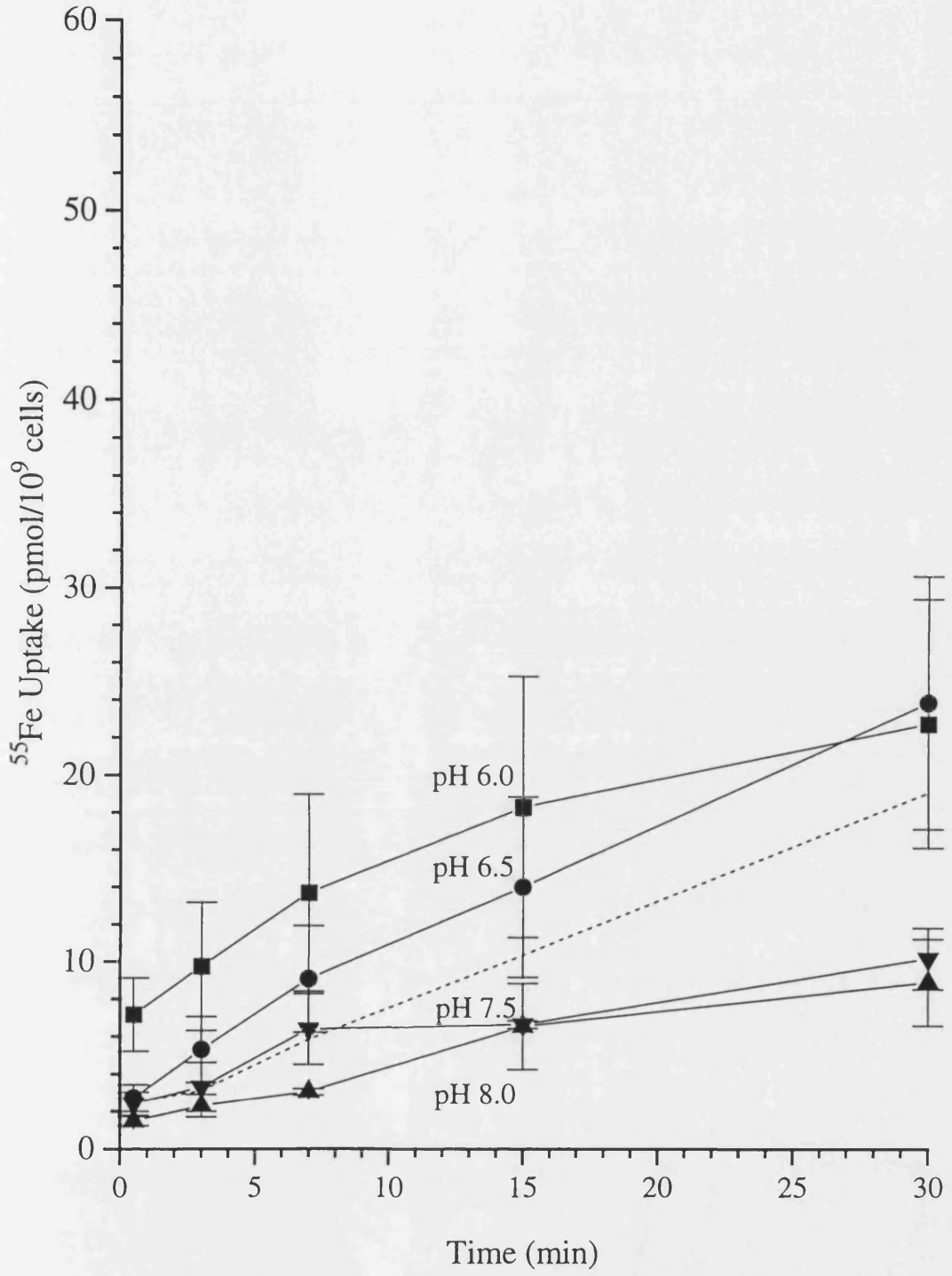


Figure 5.4. *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* PAO1 grown in succinate medium. Four different uptake media were used at pH 6.0, 6.5, 7.5 and 8.0. The uptake media contained *myo*-$InsP_6$ (100 μM), $^{55}FeCl_3$ (200 nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0.

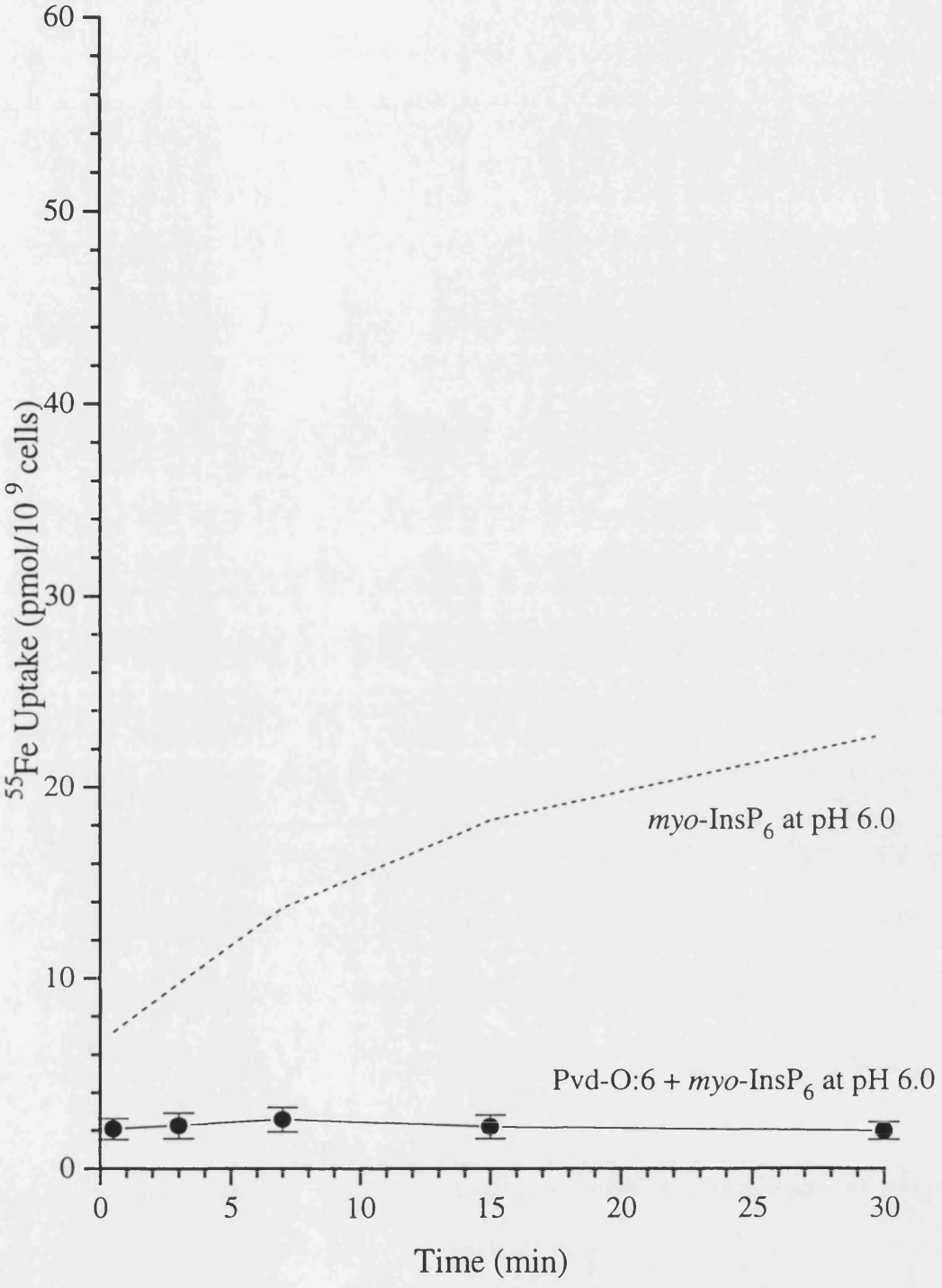


Figure 5.5. The combination of *myo*-InsP6 and 0:6-Pvd-mediated iron uptake in *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium, adjusted to pH 6.0, contained *myo*-$InsP_6$ (100μM), 0:6 Pvd (200μM), $^{55}FeCl_3$ (200nM), glucose (60μM) and 1 ml of cells OD_{470} 1.0. The dashed line is duplicated from figure 5.4 for comparison.

5.5. Discussion

In dilute aqueous solution, *myo*-$InsP_6$ assumes the 5e/1a conformation (section 4.6.) which is thought to afford *myo*-$InsP_6$ the ability to inhibit completely Fe(III) catalysed hydroxyl radical formation at 100 μM. Spiers *et al.*, (1996) also noted complete inhibition of the reaction using *myo*-Ins(1,2,3)P_3 suggesting that this effect was due primarily to the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif forming the correct conformation for optimal iron chelation. In addition, Hawkins *et al.*, (1993), when studying inositol pentakisphosphates, noted that only those compounds possessing the 1,2,3 (equatorial, axial, equatorial) trisphosphate motif were capable of inhibiting this reaction. Similarly, it was noted that of a range of inositol tetrakisphosphates tested, only Ins(1,2,3,5)P_4 and Ins(1,2,3,4)P_4 were able completely, or almost completely, to inhibit hydroxyl radical generation when present at 100 μM. In contrast, Ins(1,2)P_2 was unable to inhibit hydroxyl radical generation (Spiers *et al.*, 1996).

In this chapter, and in common with iron transport data, the inositol trisphosphates varied greatly in their ability to inhibit free radical generation. *myo*-Ins(1,2,3)P_3 caused considerable, although not complete, inhibition of hydroxyl radical generation. *myo*-Ins(1,4,6)P_3 was an effective inhibitor of Fe(III)-catalysed hydroxyl radical generation whereas *myo*-Ins(1,3,4)P_3 was unable to cause substantial inhibition. *myo*-Ins(1,4,5)P_3 caused a slight potentiation of hydroxyl radical generation although the possible biological consequences of enhanced hydroxyl radical generation are unknown.

The inositol tetrakisphosphates *myo*-Ins(1,2,4,5)P_4 and 3,6 di-O-benzoyl *myo*-Ins(1,2,4,5)P_4 were tested for their ability to inhibit Fe(III)-catalysed hydroxyl radical generation. Interestingly, there was little difference between the ability of these two compounds to inhibit the reaction despite being considerably different in their abilities to mediate iron transport. Studies by Spiers *et al.*, (1996) have shown that Ins(1,3,4,5)P_4 and Ins(1,2,4,6)P_4 produced 70% and 80%, respectively, of the formaldehyde produced by the control.

Using these observations, it is possible to propose a model to demonstrate the manner in which inositol phosphates interact with Fe(III). In order to catalyse hydroxyl radical formation, iron must have one or more free co-ordination sites or have co-ordination sites occupied by a readily dissociable ligand such as water. As noted previously, the ability of *myo*-Ins(1,2,3)P_3 and *myo*-InsP_6 to inhibit iron-catalysed hydroxyl radical formation is thought to be due to the 1,2,3 trisphosphate motif providing an optimal binding arrangement (Hawkins *et al.*, 1993). A model proposing the mechanism of interaction of Fe(III) with the 1,2,3 trisphosphate motif is illustrated which assumes the inositol phosphate to be in the 5a/1e conformation. Consequently, this model suggests that the important component is the axial, equatorial, axial 1,2,3 trisphosphate arrangement rather the previously proposed equatorial, axial, equatorial arrangement. In the absence of cations, this conformation is thought to be sterically unfavourable although Isbrandt and Oertel (1980) suggested that the alkali metals preferentially bind to, and stabilise the 5a/1e arrangement. Similarly, addition of Fe(III) is proposed to have similar stabilising effects.

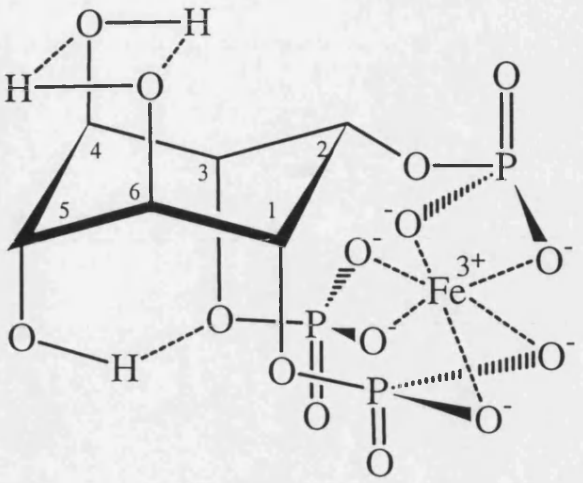


Figure 5.6. A diagrammatic model of the proposed interaction between Fe(III) and *myo*-Ins(1,2,3)P_3

The proposed model for interaction between Fe(III) and the 1,2,3 trisphosphate motif is based on Fe(III) possessing 6 co-ordination sites to form an octahedral complex (Figure 5.6). It can be seen that the six oxygen anions of the 1,2,3 trisphosphate motif of *myo*-Ins(1,2,3)P_3 interact with the 6 co-ordination sites of Fe(III). This results in the formation of a stable complex with no co-ordination sites being available to interact with the hydrogen peroxide of the hydroxyl radical assay. In addition, the hydroxyl groups in positions 4-, 5- and 6- may take part in hydrogen bonding hence stabilising the conformation of the inositol phosphate ring. This makes iron release from this compound to a putative carrier system particularly difficult.

A model is also proposed to indicate the mechanism by which *myo*-Ins(1,4,5)P_3 is capable of potentiating the hydroxyl radical assay. Again, this model assumes the 5e/1a conformation of the compound and is illustrated below.

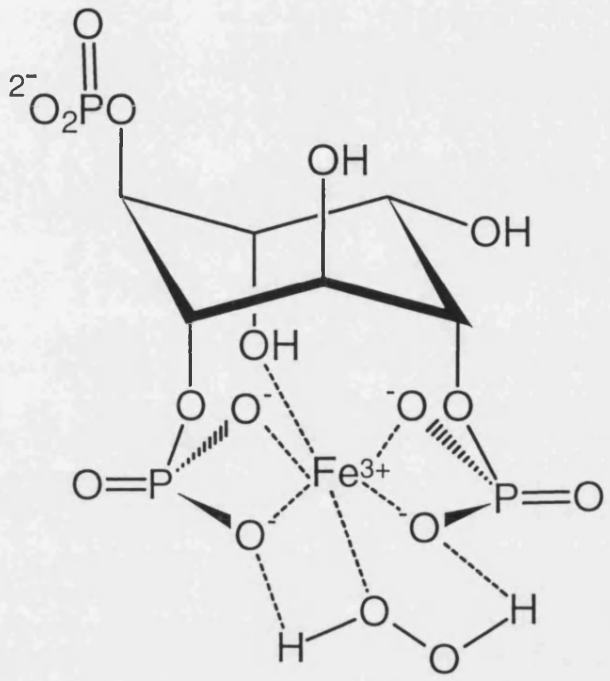


Figure 5.7. A diagrammatic model of the proposed interaction between Fe(III) and *myo*-Ins(1,4,5)P_3

This model proposes that the site of interaction with iron is between the 1- and 5-position phosphate groups rather than the previously suggested 4,5 bisphosphate motif. Whilst the equatorial 4,5 bisphosphate arrangement in the 5e/1a conformation may be important in eukaryotic receptor recognition, it appears that it is the axial 1- and 5- position phosphates that are important in this model. Interestingly, the oxygen anions of these two phosphate groups only occupy 4 co-ordination sites of Fe(III)

leaving two free sites. One site is probably occupied through interaction with the 3-position hydroxyl group leaving one free co-ordination site that is probably associated with an easily dissociable ligand such as water. This site therefore appears suitable for interaction with hydrogen peroxide of the hydroxyl radical system. In addition, the hydrogen peroxide is capable of undergoing hydrogen bonding to oxygen atoms of the 1- and 5-position phosphate groups which may potentiate hydroxyl radical generation. Interestingly, this compound was the most efficient mediator of iron transport and may illustrate the ease with which this compound yields iron to a putative iron transport system.

This model also explains the slight inhibition of the hydoxyl assay observed with the tetrakisphosphates *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,3,4,5)P_4. With these compounds, addition of an extra phosphate group provides additional negatively charged oxygen anions which are capable of interacting with co-ordination sites of Fe(III). This may explain the lower iron uptake profiles associated with these compounds in comparison to *myo*-Ins(1,4,5)P_3. Because the principle site of interaction is *via* the functional groups at the 1-, 3- and 5- positions, and the 2-position group is less involved, the model also explains why the *scyllo* derivatives of Ins(1,2,4,5)P_4 and Ins(1,3,4,5)P_4 mediate similar iron profiles to the *myo* derivatives.

The model also explains why *myo*-Ins(1,2,4,5)P_4 and 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 appear to have similar abilities to interact with iron. Whilst the substitution of the hydroxyl group for the large *O*-benzoyl group at the 6 position should be of little consequence, removing the 3-position hydroxyl group may be

considered as detrimental. However, the charge associated with carbonyl group may interact with the co-ordination site of Fe(III) in a similar manner to the oxygen of the hydroxyl group. Consequently, the low iron transport profile associated with 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 is probably a result of the large *O*-benzoyl groups reducing the ability of the compound to interact with the bacterial surface rather than reflecting a difference in ability to interact with iron.

This may also explain why *myo*-Ins(1,3,4,6)P_4 is capable of chelating iron within this system whereas 2,5 di-*O*-methyl *myo*-Ins(1,3,4,6)P_4 is not. All inositol phosphate-iron interactions have depended upon the interaction of at least two phosphate groups and one other charged group such as a hydroxyl group or the carbonyl bond. With 2,5 di-*O*-methyl *myo*-Ins(1,3,4,6)P_4 there is very little charge associated with the *O*-methyl arrangement which may result in the inability of this compound to chelate iron.

myo-Ins(1,3,4)P_3 is also capable of slightly inhibiting the hydroxyl assay. In common with *myo*-Ins(1,4,5)P_3, this has two axial phosphates and one axial hydroxyl group with which to interact with the co-ordination sites of Fe(III). However, *myo*-Ins(1,3,4)P_3 also has the equatorial 2-position hydroxyl group which may also be capable of interacting with one of the co-ordination sites of Fe(III) hence causing a slight inhibition of the reaction. The iron transport profile associated with this compound is lower than expected and the reason for this is unclear. However, it may be a result of this compound presenting iron to the bacterial surface in a manner not easily utilised by the bacterium. The remaining compounds, *myo*-Ins(1,4,6)P_3

and *myo*-Ins(1,2,4,6)P_4 remain the two anomalies of this study. *myo*-Ins(1,4,6)P_3 is particularly efficient at inhibiting iron-catalysed hydroxyl radical generation yet is an efficient mediator of iron transport. When examining the compound in either the 5e/1a or 5a/1e conformation, the model does not explain why this compound is particularly effective at inhibiting the reaction. Furthermore, a compound with such an ability to interact with iron would not be expected to be an efficient mediator of iron transport. This situation is even more confusing with respect to *myo*-Ins(1,2,4,6)P_4. Addition of the phosphate group to the 2-position appears to reduce the ability of this compound to interact with iron, yet the iron uptake profile associated with this compound is indicative of a compound with much greater ability to interact with iron.

The assumptions regarding the abilities of these compounds to bind iron and then subsequently mediate iron transport into *P. aeruginosa* PAO1 were given further strength by a series of competition iron transport experiments. These experiments using equimolar concentrations of one inositol phosphate associated with high iron transport and a low ability to interact with iron, and another associated with low iron transport and a greater ability to interact with iron, resulted in an iron transport profile associated with the compound that had the greater ability to interact with iron. For example, an equimolar mixture of *myo*-Ins(1,2,3)P_3 and *myo*-Ins(1,4,5)P_3 resulted in an uptake profile considerably lower than that associated with *myo*-Ins(1,4,5)P_3 alone. However, in this example, uptake was not as low as that associated with *myo*-Ins(1,2,3)P_3 alone. A mixture of *myo*-InsP_6 and *myo*-Ins(1,4,5)P_3 resulted in an iron uptake profile that was almost identical to that

achieved using *myo*-$InsP_6$ alone. The greater ability of *myo*-$InsP_6$ to reduce the uptake profile associated with *myo*-Ins(1,4,5)P_3 is perhaps an indication that this compound has a greater affinity towards iron than *myo*-Ins(1,2,3)P_3.

These data indicate that measuring the ability of these compounds to inhibit iron-catalysed hydroxyl radical generation gives some indication towards binding affinity. However, there are some notable exceptions. It therefore appears that those compounds with the greater affinity towards iron are less able to mediate iron uptake into *P. aeruginosa*, perhaps as result of a reduced ability to yield iron to a putative carrier system.

The observation that *myo*-$InsP_6$ is able to compete successfully with *myo*-Ins(1,4,5)P_3 to mediate iron transport prompted futher competition studies using a natural siderophore. The aim of these experiments was to assess the ability of *myo*-$InsP_6$-mediated iron transport to confer a competitive advantage in the environment. Iron transport assays were initially performed using a combination of *myo*-$InsP_6$ and Pvd:06 (pyoverdine produced by *P. aeruginosa* S.T:06) in which *myo*-$InsP_6$ was unable to compete with the naturally ocuuring pyoverdine. This again supports the hypothesis regarding competition for iron, as pyoverdine has an estimated affinity for iron at neutral pH of 10^{32} M^{-1} (Demange *et al.*, 1987), whereas for *myo*-$InsP_6$, this has been estimated to be in the range of 10^{25}-10^{30} M^{-1} (Hawkins *et al.*, 1993). However, these experiments did not take into account the fact that *P. aeruginosa* is found in various evironments which may not necessarily be at neutral pH. *myo*-$InsP_6$ is a highly charged molecule possessing six phosphate groups that are capable of

existing in various states of ionistation depending on the pH of the environment hence a series of iron transport experiments were performed at varying pH. The p*K*a values for each of the phosphate groups around *myo*-$InsP_6$ are illustrated below and indicate the sequential transition of each phosphate group from the free acid to the mono-anion and ultimately to the di-anion.

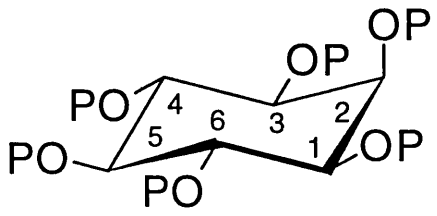


Figure 5.8. *myo*-Inositol hexakisphosphate illustrating the numbering of the inositol ring

Position of Phosphate Group of *myo*-$InsP_6$	pKa (Dissociation to Mono-anion)	pKa (Dissociation from Mono- to Di-anion)
C-1	1.5	5.7
C-2	1.1	6.85
C-3	1.5	12.0
C-4	2.1	10.0
C-5	1.7	7.6
C-6	2.1	10.0

Table 5.3. p*K*a values for the six phosphate groups of *myo*-$InsP_6$ for the dissociation from free acid to the mono-anion and subsequent dissociation from the mono- to bi-anion (Costello *et al.*, 1976).

Table 5.2 illustrates that conversion of each of the phosphate groups from the free acid to the mono-anion occurs over a narrow pH range. All six phosphate groups exist as the mono-anion at a relatively low pH. However, differences become apparent when considering conversion from the mono-anion to the di-anion which occurs over a broad pH range. For example, the 1-position phosphate has a p*K*a of 5.7 for dissociation to the di-anion whereas the 3-position phosphate group has a mono- to di-anion p*K*a of 12.0. Iron transport assays were performed at pH 6.0, 6.5, 7.5 and 8.0. Table 5.2. shows that over this pH range, there is some variation of ionisation around the *myo*-$InsP_6$ molecule.

The results from transport assays performed at different pH values are illustrated in figure 5.4. There is very little difference between iron transport at pH 6.0 and pH 6.5. This is expected because at both these pH values only the 1-position phosphate group has started to undergo transition to the di-anion. However, increasing the pH from 6.5 to 7.0 appears to reduce iron transport which mirrors the increased dissociation of the 2-position phosphate group. A further increase in pH from 7.0 to 7.5 results in a further reduction of iron transport as the 5-position phosphate group undergoes dissociation. Between pH 7.5 and pH 8.0, there is little difference in iron transport which is probably a result of similar degrees of ionisation. Clearly, reducing the pH reduces the extent of ionisation of *myo*-$InsP_6$ which consequently appears to increase iron transport.

The observation that *myo*-$InsP_6$ transport was enhanced at pH 6.0 in comparison to pH 7.0, prompted competition experiments with Pvd:06 at the lower pH value. The

dissociation constants for the acidic groups of Pvd:06 are not available although those of pyoverdine PaA have been published (Albrecht-Gary *et al.*, 1994). Whilst there are differences in structure between these two pyoverdines, PaA can be used as a guide to predict the level of ionisation of Pvd:06. The p*K*a values for each acidic group of pyoverdine PaA are illustrated below.

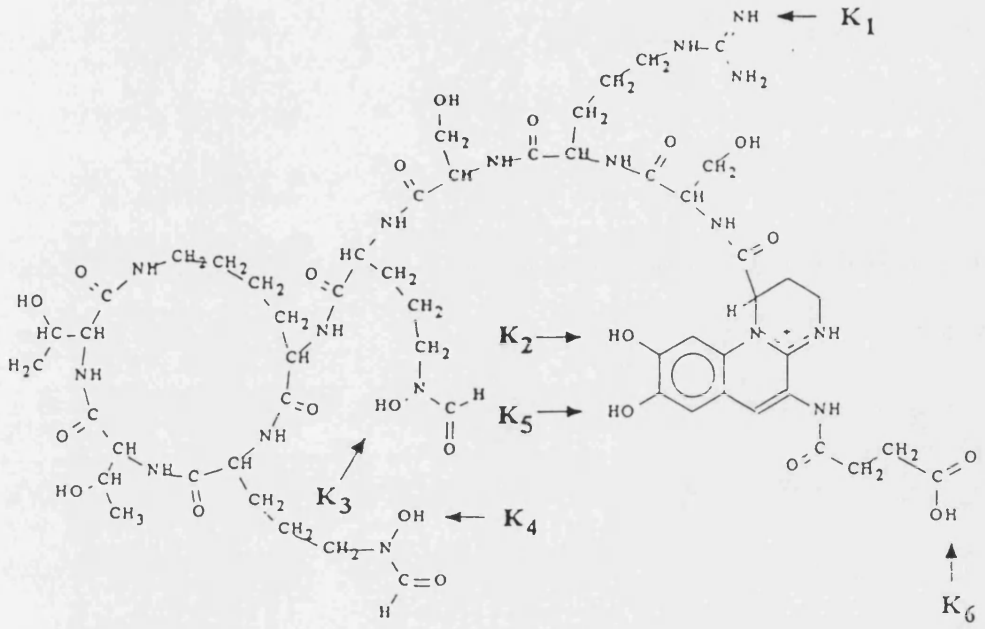


Figure 5.9. The structure of Pyoverdine PaA illustrating the positions of the six acidic groups (Albrecht-Gary *et al.*, 1994).

Acidic Group Number	p*K*a
K_1	12.2
K_2	10.8
K_3	7.6
K_4	8.6
K_5	5.7
K_6	4.8

Table 5.4. p*K*a values for each of the acidic groups around Pyoverdine PaA (Albrecht-Gary *et al.*, 1994).

Table 5.4 illustrates that at both pH 7.0 and pH 6.0, pyoverdine PaA possesses two dissociated acidic groups (K_5 and K_6) hence reducing the pH from 7.0 to 6.0 should make little difference to the iron chelating ability of the molecule. Iron transport assays at pH 6.0 illustrate that the less ionised version of *myo*-$InsP_6$ was still unable to compete with Pvd:06 (figure 5.5).

These results give further support to the theory that reducing the affinity of the compound towards iron results in enhanced iron uptake. When considering *myo*-$InsP_6$, reducing the pH of the medium reduces the charge associated with the compound resulting in a lower affinity towards Fe(III). In common with other compounds with a lower affinity towards Fe(III), e.g. *myo*-Ins(1,4,5)P_3, this results in an enhanced iron uptake. However, because the enhanced *myo*-$InsP_6$-mediated uptake at pH 6.0 is based on a reduced affinity towards iron whilst the extent of

ionisation of the pyoverdine molecule appears unchanged, it appears even less likely that *myo*-$InsP_6$ is able to compete with Pvd:06.

In summary, this chapter has attempted to relate the ability of several inositol phosphates to mediate iron transport in relation with their ability to interact with iron. A model was proposed to explain the apparent inverse relationship between the ability to mediate iron transport and the ability to interact with ferric iron. In general, it appears that increasing the affinity of a compound towards iron results in a reduced ability to mediate iron transport into *P. aeruginosa* PAO1. The clearest examples of this phenomenon include *myo*-$InsP_6$ and *myo*-$Ins(1,2,3)P_3$ where it appears that the presence of the 1,2,3 (axial, equatorial, axial) motif confers an enhanced ability to interact with iron and a low iron transport profile. In contrast the 1,5-bisphosphate motif appears capable of interacting with iron in such a manner that allows interaction with hydrogen peroxide in the hydroxyl radical assay. Compounds containing this motif, namely *myo*-$Ins(1,4,5)P_3$, *myo*-$Ins(1,2,4,5)P_4$ and *myo*-$Ins(1,3,4,5)P_4$, appear to have lower affinities towards iron yet are considerably better mediators of iron transport. Such differences may highlight the relative abilities of these compounds to yield iron to a putative carrier system. Finally, *myo*-$InsP_6$-mediated iron transport does not confer a competitive advantage in an environment where non-utilisable pyoverdines are present.

Having determined several structure-iron transport relationships and related these to the ability of these compounds to interact with ferric iron, chapter 6 attempts to

determine the mechanisms by which iron complexed with inositol phosphates is liberated to the bacterium.

Chapter 6

Iron Release From Inositol Phosphates

6.1. Introduction.

Previous chapters provided an insight into the relationships between the ability of several inositol phosphates to interact with iron and their abilities to mediate iron transport into *P. aeruginosa* PAO1. Whilst there are some exceptions, it appears that the greater the affinity of a particular compound towards Fe(III) the less able it is to mediate iron transport. The aim of this chapter is to determine the mechanisms by which iron is liberated from inositol phosphates and made available to the bacterium.

The mechanisms by which endogenous siderophores yield iron to the bacterium are still subject to debate. Research into enterobactin-mediated iron uptake in *E. coli* has suggested two mechanisms, one of which involves the action of a cytoplasmic esterase, which cleaves the siderophore inside the bacterium hence releasing the iron (O'Brien *et al.*, 1970). However, the discovery that certain hydrolysis-resistant synthetic siderophores could still release iron led to a heightened interest in other mechanisms (Heidinger *et al.*, 1983). Consequently, it was suggested that reductases may reduce Fe(III) to Fe(II) with a subsequent release of iron from the siderophore (Fischer *et al.*, 1990). In *P. aeruginosa*, reductase activity has been implicated in the release of iron from the ferripyochelin complex, the ferricitrate complex (Cox, 1980) and the ferripyoverdine complex (Halle and Meyer, 1992a). It was believed that this

system utilised specific enzymes although it now appears that all of the above operate *via* a common non-specific pathway (Halle and Meyer, 1992b).

In this study, three mechanisms of iron release were studied. Firstly, a conformationally restricted inositol phosphate, characterised by its inablity to change ring conformation, was used. Secondly, attempts were made to determine whether phytases cleave phosphates from the inositol ring with a subsequent release of iron and finally, a series of experiments were performed to determine whether reductase activity was capable of mediating iron release from inositol phosphates.

6.2. Inositol Phosphate-Mediated Iron Transport Using a Cyclic Phosphate Analogue of *scyllo*-Inositol (1,4,5) Trisphosphate

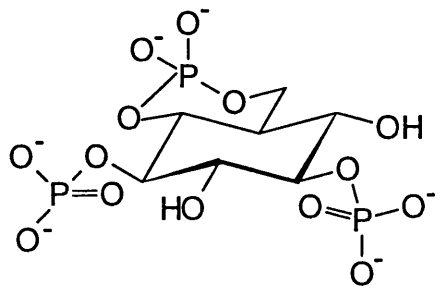


Figure 6.1. Cyclic Phosphate Analogue of *scyllo*-Inositol (1,4,5) Trisphosphate

(D/L)-deoxy-6-hydroxymethyl-*scyllo*-inositol 1:7-cyclic,2,4-trisphosphate is a structurally complex compound. It was designed to examine the activity surrounding the 4,5 bisphosphate motif and to determine the effect of preventing conformational

mobility of *myo*-Ins(1,4,5)P_3 (Riley and Potter, 1995). This compound is essentially a *scyllo*-Ins(1,4,5)P_3 derivative that has been tethered *via* the 4-position phosphate group to the equivalent carbon of the 3-position using a methylene group. Unlike *myo*-Ins(1,4,5)P_3, this compound is unable to change conformation from the 5e/1a to the 5a/1e conformation.

Eukaryotic calcium mobilisation studies indicated that despite both conformational restriction and charge reduction at the highly sensitive 4,5-bisphosphate motif, this compound was able to behave as a full agonist. However, this was at an EC_{50} around 40-fold higher than *myo*-Ins(1,4,5)P_3 (Riley and Potter, 1995).

Iron transport assays were performed using this compound to indicate whether conformational change plays a role in the ability of inositol phosphates to release iron to *P. aeruginosa* PAO1. The results are illustrated in figure 6.2 (p131) which indicate that this compound retains some iron transport activity although less than that achieved using *myo*-Ins(1,4,5)$_3$. The initial rate of iron-uptake was 22.22 pmol/min/10^9 cells and the final amount of iron accumulated was 32.55±2.11 pmol/10^9 cells after 30 minutes.

6.3. The Role of Phytases in Inositol Phosphate-Mediated Iron Transport

Many organsisms use *myo*-InsP_6 (phytate) as a phosphate source *via* enzymatic cleavage of phosphate groups from the inositol ring. Such phosphate cleaving enzymes have been termed phytases and two classes of phytases are recognised. 3-phytase catalyses the removal of the D-3-phosphate from phytic acid and 6 phytase

attacks the D-6 phosphomonoester. (Loewus, 1990; Gibson and Ullah, 1990). Both enzymes are capable of causing successive dephosphorylation of inositol pentakisphosphates to free inositol. The 3-phytases are found typically in micro-organisms and filamentous fungi and the 6-phytase is the dominant plant enzyme. Phytases have been identified in *Pseudomonas* spp (Irving and Cosgrove, 1971) which do not appear to require substrate induction. There has been a recent resurgence in phytase research particularly within agriculture. It has been found that dietary supplementation with phytase optimises phosphate digestion from animal feed hence allowing the use of less expensive, low-grade feed and reducing manure-derived phosphate output into the environment. Research has focused on the addition of *Aspergillus niger* phytase to the feeds of piglets, broiler chickens, carp and rainbow trout (Mitchell and Edwards, 1996; Kirchgessner and Windisch, 1995; Schafer *et al.*, 1995; Rodehatscord and Pfeffer, 1995).

In humans, addition of *Aspergillus niger* phytase to the diet also increases phosphate and iron absorption and appears to work well at the low pH encountered in the human stomach (Sandberg *et al.*, 1996). However, whilst the presence of additional phytase may improve iron absorption, it may also remove the protective effect of phytate increasing the risk of developing colonic cancer (Iqbal *et al.*, 1994).

In order to determine whether *Pseudomonas* phytase may play a role in inositol phosphate-mediated iron transport, iron transport assays were performed using the phosphorothioate derivative of *scyllo*-Ins(1,2,4,5)P_4, *scyllo*-Ins(1,2,4,5)PS_4, The phosphate groups of *scyllo*-Ins(1,2,4,5)PS_4 are linked to the carbon ring *via* sulphur

atoms rather than the usual oxygen atom hence conferring phytase-resistant phosphorothioate groups. The rationale behind using this compound is that if it was able to chelate iron yet not mediate iron transport, then this may be a result of phytases being unable to cleave the phosphorothioate bond preventing iron release to the bacterium. The structure of *scyllo*-Ins(1,2,4,5)PS_4 is illustrated below.

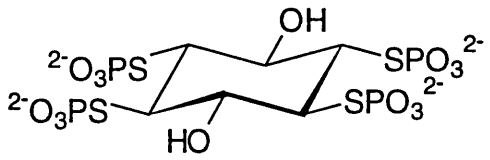


Figure 6.3. *scyllo*-Ins(1,2,4,5)PS_4

Unfortunately, it was not possible to achieve solubilisation of iron in this system as precipitation of the ^{55}Fe always occurred. It was noted that a faint yellow colouration was also observed suggesting precipitation of the phosphorothioate compounds. This was observed even in the absence of cells. Consequently, the potential role of phytase in inositol phosphate-mediated iron transport remains unresolved.

6.4. The Role of Reductases in Inositol Phosphate-Mediated Iron Transport

As noted in the introduction, whilst much is known about siderophores and receptor production in *P. aeruginosa*, comparatively little is known regarding the mechanism whereby iron is released from the siderophore itself. However, it has been noted that several species of bacteria produce reductase enzymes that are thought to reduce Fe(III) to Fe(II) which is only weakly bound to the siderophore. This change in

valency is thought to be responsible for the release of iron to the interior of the bacterium.

It was originally thought that the reductase enzyme reduced directly the ferrisiderophore complex. However, it appears that the reaction relies upon an NADH/FMN oxidoreductase system thought to involve a chemical reduction by $FMNH_2$ (Halle and Meyer, 1992b). It is thought that the reaction is initiated by the enzyme-catalysed reduction of FMN to $FMNH_2$ by NADH. Next is the $FMNH_2$-mediated reduction of Fe(III) to Fe(II). The amount of Fe(II) produced can be quantified by its ability to form a coloured Fe(II)-Ferrozine complex which is followed spectrophotometrically. Evidence for this mechanism is provided by the observation that FMN is an essential component in the reaction. In addition, the reaction is inhibited by the presence of oxygen. It was thought that the inhibitory effect of oxygen was due to reoxygenation of Fe(II) to Fe(III), thus competing with the Fe(II)-Ferrozine formation (Straka, 1979). However, in light of the $FMNH_2$ mechanism, it appears that oxygen inhibition is a result of oxidation of the $FMNH_2$ (Halle and Meyer, 1992b).

The reactions are summarised below.

$$FMN + 2\ NADH \rightarrow FMNH_2 + 2\ NAD^+$$

$$2\ Fe^{3+} + FMNH_2 \rightarrow 2\ Fe^{2+} + FMN + 2H^+$$

$$Fe^{2+} + \text{Ferrozine} \rightarrow \text{Fe(II)-Ferrozine (Abs 562 nm)}$$

This assay was used to measure the ability of *P. aeruginosa* PAO1 reductase to reduce iron bound to *myo*-$InsP_6$ and a variety of other inositol phosphates. Figure 6.4 (p132) illustrates the NADH/FMN reduction of Fe(III) bound to *myo*-$InsP_6$. The bacterial extract, mixed with ferri-*myo*-$InsP_6$, was sparged with argon for 10 minutes and the reaction started by addition of NADH, FMN and Ferrozine. Anaerobic conditions were maintained by a constant flow of argon through the spectrophotometer cell. There was evidence of a slight lag phase, probably due to small amounts of residual oxygen in the system, followed by an increase in OD_{562} over 30 min (figure 6.4). Also illustrated is the reduction of ferripyoverdine (0.2 mM) which results in notably less Fe(II)-Ferrozine production than that achieved using ferri-*myo*-$InsP_6$. The assay was also performed in aerobic conditions which inhibited the reaction although the slight increase in Fe(II)-Ferrozine production after 25 min may be due to eventual oxygen depletion. Similarly, replacement of bacterial extract with Buffer A (Section 3.11) resulted in almost complete inhibition of the reaction. Figure 6.4. also illustrates that reduction of ferri-*myo*-$InsP_6$ is an FMN-dependent reaction.

Reductase assays were performed using a variety of inositol phosphates, and in all cases, reduction was dependent on the presence of bacterial extract i.e. there was no self reduction. Figure 6.5. (p133) illustrates Fe(II)-Ferrozine formation following reduction of ferri-*myo*-Ins(1,3,4,5)P_4, ferri-*myo*-Ins(1,2,4,6)P_4 ferri-*myo*-Ins(1,2,4,5)P_4 and ferri-3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4. It is interesting to note that there is very little difference between these compounds in their abilities to liberate iron following reductase activity. Also of interest are the data obtained using

inositol trisphosphates (figure 6.6 - p134). Assays using *myo*-Ins(1,3,4)P_3, *myo*-Ins(1,4,5)P_3 and *myo*-Ins(1,4,6)P_3 indicated very little difference between them, all three liberated iron in the presence of reductase to a similar extent. However, *myo*-Ins(1,2,3)P_3 resulted in notably less iron being liberated to the assay with the amount of Fe(II)-Ferrozine being almost the same as that achieved using *myo*-InsP_6. Due to the limited supply of these compounds, all assays represent n=1 with the exception of *myo*-InsP_6 where n=3.

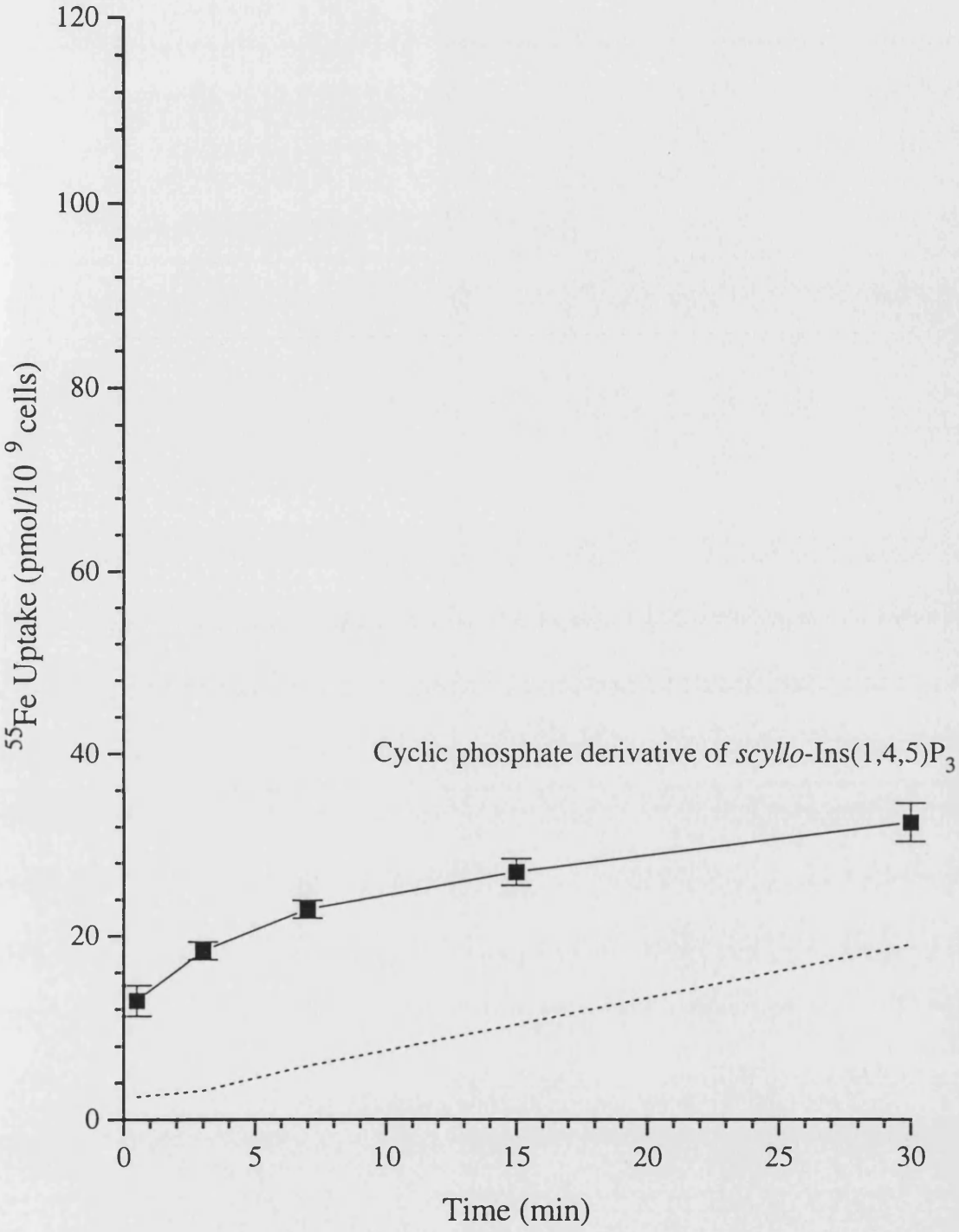


Figure 6.2. Cyclic phosphate derivative of *scyllo*-Ins(1,4,5)P_3-mediated iron transport in *P. aeruginosa* PAO1 grown in succinate medium. The uptake medium contained the cyclic phosphate derivative of *scyllo*-Ins(1,4,5)P_3 (100μM), $^{55}FeCl_3$ (200nM), glucose (60μM) and 1 ml of cells at OD_{470} 1.0. The dashed line represents myo-InsP_6-mediated iron transport and is duplicated from Fig 4.2. for comparison.

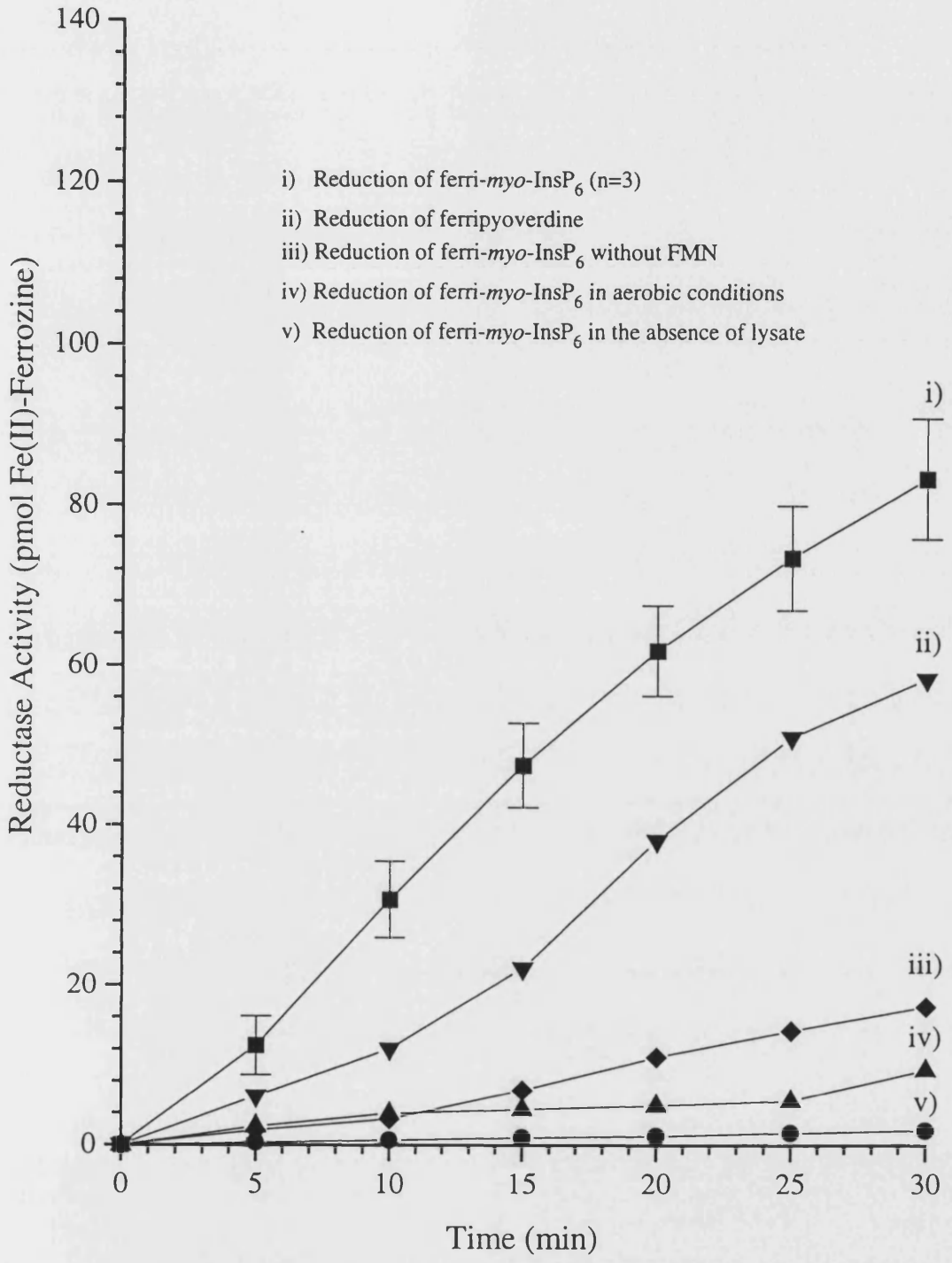


Figure 6.4. Anaerobic reductase activity of *P. aeruginosa* lysate. Each 2 ml assay contained 1.6 mg protein, inositol phosphate or pyoverdine (0.2 mM), $FeCl_3$ (0.2 mM), Ferrozine (0.8 mM), NADH (0.15mM) and FMN (0.05mM).

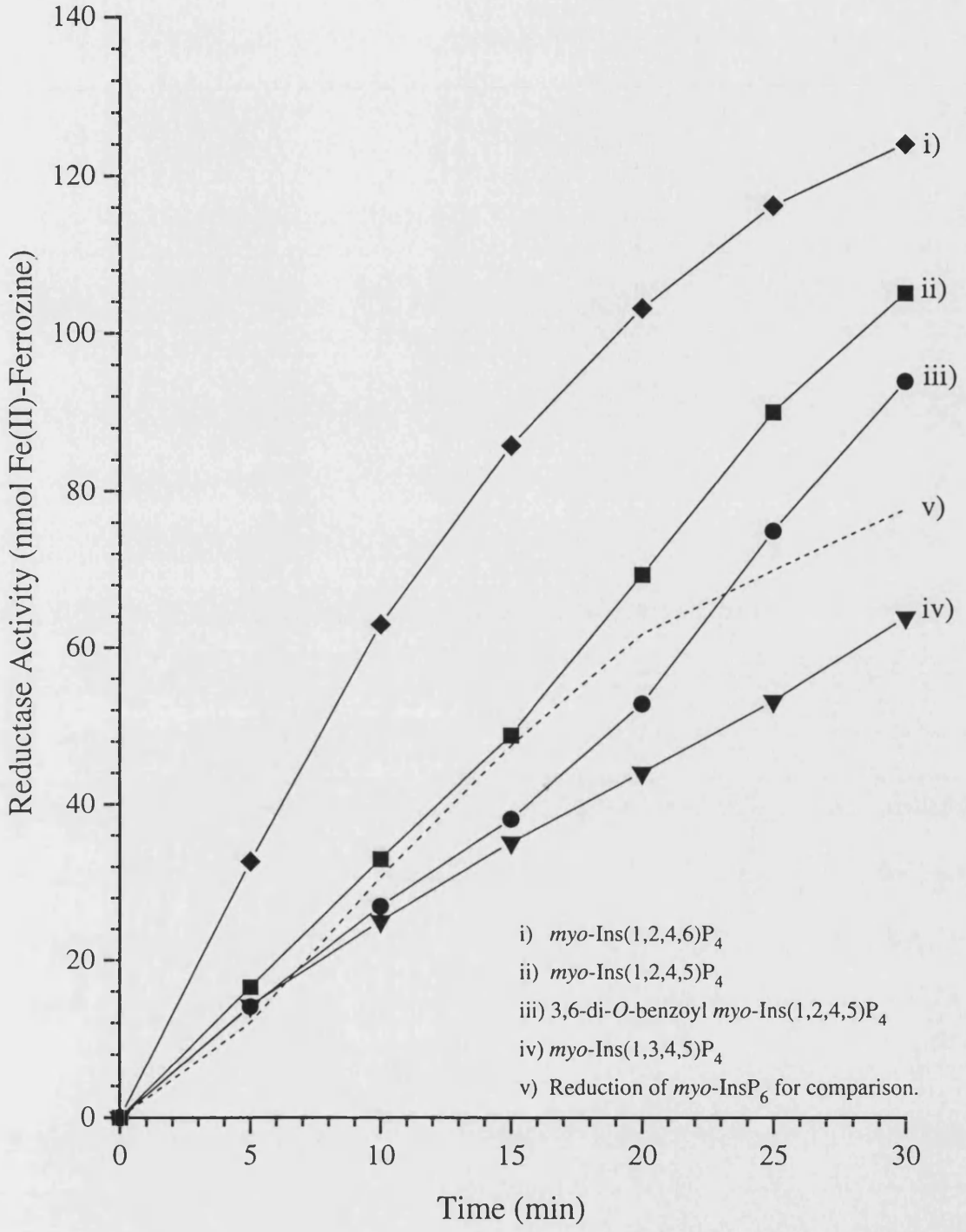


Figure 6.5. Anaerobic reductase activity of *P. aeruginosa* lysate. Each 2 ml assay contained 1.6 mg protein, inositol phosphate (0.2 mM), $FeCl_3$ (0.2 mM), Ferrozine (0.8 mM), NADH (0.15 mM) and FMN (0.05 mM).

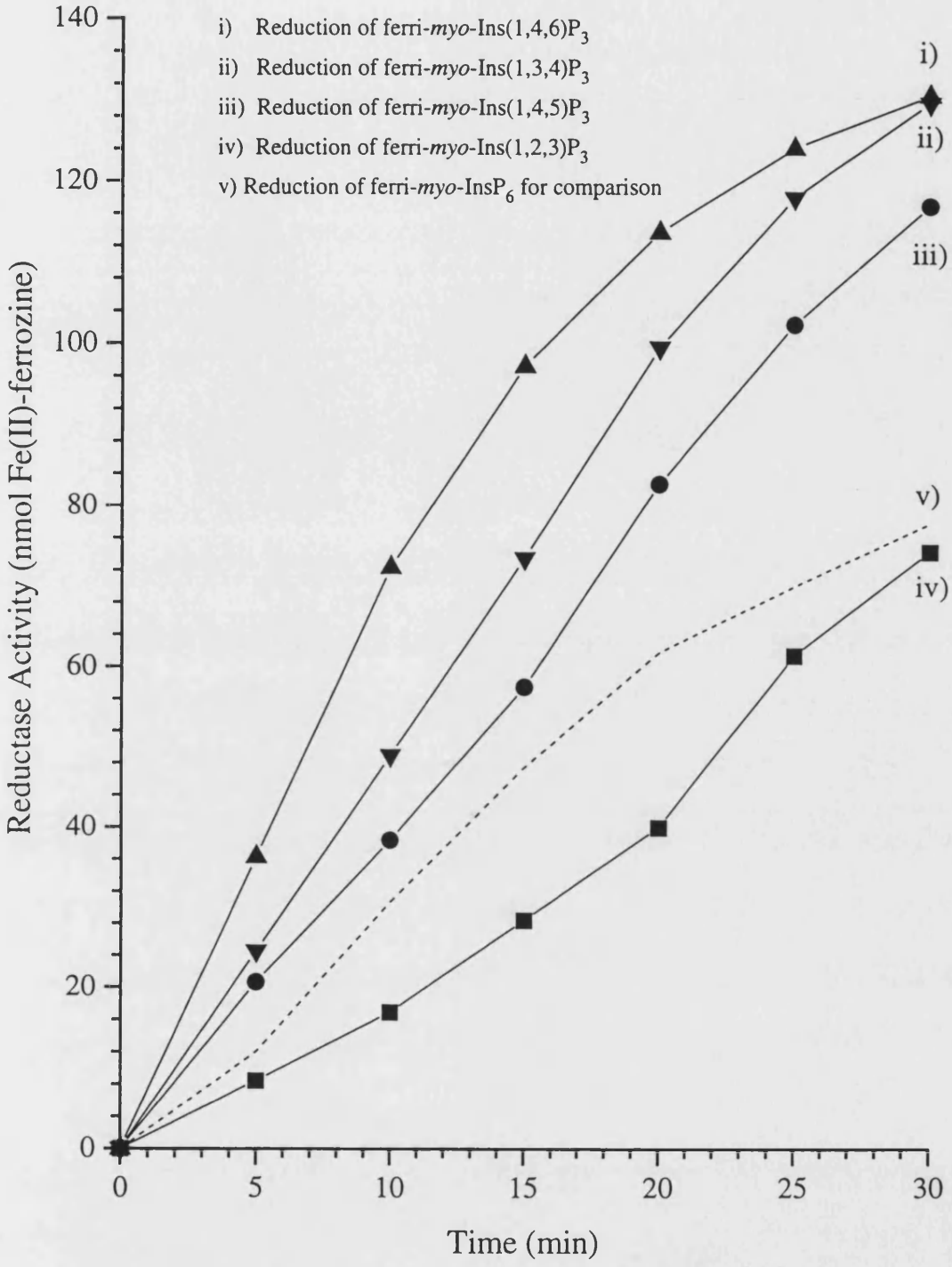


Figure 6.6. Anaerobic reductase activity of *P. aeruginosa* lysate. Each 2 ml assay contained 1.6 mg protein, inositol phosphate (0.2 mM), $FeCl_3$ (0.2 mM), Ferrozine (0.8 mM), NADH (0.15 mM) and FMN (0.05mM)

6.5 Discussion

This chapter has made progress in developing an insight into the mechanisms by which inositol phosphates liberate iron to the bacterium. The use of a conformationally restricted analogue of *scyllo*-Ins(1,4,5)P_3 gave an indication that a change in ring conformation is not essential for inositol phosphate-mediated iron transport. The ability of this compound to chelate iron may be a result of there being sufficient mobility of the equatorially arranged 1- and 5-position phosphate groups with additional interaction from the 6-position hydroxyl group. Alternatively, interaction with iron may occur *via* a combination of the modified 4,5 motif and the equatorially arranged 6-position hydroxyl group. Similarly, conformational restriction was not a barrier to the ability of this compound to mediate calcium mobilisation in eukaryotic systems which may reflect some retention of activity surrounding the 4,5 motif (Riley and Potter, 1995).

It was unfortunate that it was not possible to assess the role of phytase activity. The fact that the cells used in iron transport assays were grown in phosphate-rich succinate medium should be of little consequence as Irving and Cosgrove (1971) noted that *Pseudomonas* phytase production did not require substrate induction. The appearance of precipitated iron sulphide and the subsequent ^{55}Fe(III) precipitation suggested that the phosphorothioate analogue of *scyllo*-Ins(1,2,4,5)P_4 had decomposed. This was most likely due to disruption of the phosphorothioate bonds linking the phosphate groups to the inositol ring.

The results obtained from the reductase assay were particularly interesting and gave further support to some key trends. It is interesting to note that reductase activity is capable of reducing Fe(III) bound to *myo*-$InsP_6$ to Fe(II) resulting in the formation of the coloured Fe(II)-Ferrozine complex. In common with previous work regarding ferripyoverdine transport (Halle and Meyer, 1992a), the reductase activity demonstrated here was dependent upon strict anaerobiosis and the presence of bacterial extract i.e. there is no self reduction of the iron.

Reductase assays using a variety of inositol phosphates produced a somewhat confusing picture. However certain key trends are apparent. The observation that pyoverdine appeared to yield less Fe(III) to the reductase system compared to *myo*-$InsP_6$, is probably indicative of the greater affinity of pyoverdine towards Fe(III). It was interesting that both *myo*-Ins(1,2,4,5)P_4 and 3,6-di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 gave similar results. It is known from previous work that these compounds have similar abilities to interact with iron yet mediate considerably different iron uptake profiles in *P. aeruginosa* PAO1. These results again suggest that the abilities of these two compounds to interact with iron are similar, hence the differences in iron uptake profiles are probably a result of the differing degrees of steric hindrance associated with these compounds.

Whilst there is little to distinguish *myo*-Ins(1,3,4)P_3, *myo*-Ins(1,4,5)P_3 and *myo*-Ins(1,4,6)P_3, there is a clear difference with *myo*-Ins(1,2,3)P_3. The degree of Fe(II)-Ferrozine formation is considerably lower with this compound and may be reflection of the 1,2,3 trisphosphate motif binding Fe(III) in such a manner that impairs

reduction to Fe(II). In this respect, *myo*-Ins(1,2,3)P_3 and *myo*-InsP_6 appear to share very similar properties, an observation noted throughout this study.

Finally there is also an interesting correlation between *myo*-InsP_6 (IP_6), *myo*-Ins(1,2,4,5)P_4 (IP_4) and *myo*-Ins(1,4,5)P_3 (IP_3). Each of these compounds is associated with increasing iron transport (IP_6<IP_4<IP_3), decreasing ability to inhibit iron-catalysed hydroxyl radical generation (IP_6>IP_4>IP_3) and increasing ability to supply Fe(III) to a reductase system (IP_6<IP_4<IP_3).

Despite reductase activity being detected in many mirco-organsims, the enzyme has only been purified in a few examples including ferri-2,3-dihydroxybenzoic acid reductase in *Bacillus subitilis* (Gaines *et al.*, 1981), and a ferrichrome reductase from *E. coli* (Fischer *et al.*, 1990). Halle and Meyer (1992a) purified a ferripyoverdine reductase in *P. aeruginosa* that was a soluble of protein of molecular weight 27,000 to 28,000 Da. Despite iron regulation of siderophore and outer membrane siderophore receptor production, reductase production was not subject to such control. Only some fungi have increased reductase activity in response to iron limitation, namely *Neurospora crassa* (Ernst, 1977) and *Saccharomyces cerevisiae* (Lesuisse and Labbe, 1989). Halle and Meyer (1992a) noted that the reductase is located cytoplasmically which implies that the ferrisiderophore complexes must become internalised prior to reduction. No enzyme activity was located in the periplasm. This study does not indicate whether the ferri-inositol phosphate complexes are internalised, a phenomenon that appears unlikely when considering the highly ionised nature of these compounds. However, a membrane bound fraction

has been found in *B. subtilis* (Gaines *et al.*, 1981) which is thought to account for 25% of reductase activity and in *E.coli*, 33% of the ferrireductase activity was associated with the membrane fraction (Fischer *et al.*, 1990).

Immunological studies examining the *P. aeruginosa* ferripyoverdine reductase suggest that it is highly conserved amongst *Pseudomonas* spp (Halle and Meyer 1992a). Similarities were observered for 18 fluorescent strains as well as the non-fluorescent strain *P. stutzeri* which produces the siderophore desferriferrioxamine E in comparison to the pyoverdines of the other strains. These results suggest that the same reductase is produced regardless of the strain or the siderophore it uses. Extending this concept further, *P. aeruginosa* reductase has been found to catalyse the reduction of ferricrocin, ferrioxamine B, ferrichrome A and ferrioxamine E (Halle and Meyer, 1992b). All of these observations further suggest the lack of specificity of this enzyme and instead lead to the suggestion that it functions as a general NADH/FMN reductase.

The lack of iron regulation of this *P. aeruginosa* NADH/FMN reductase suggests that it may have additional roles in the bacterium other than the release of iron from ferripyoverdine. Bagg and Neilands (1987a) noted that the interior of the bacterium must be highly reducing to convert Fe(III) to Fe(II) since Fe(II) is responsible for the Fe-Fur co-repressor effect. In *B. subtilis*, the FMN-dependent ferrisiderophore reductase is one of the components of the multienzyme complex involved in the biosynthesis of aromatic compounds (Gaines *et al.*, 1981; Hasan and Nester 1978). In addition, the ferrichrome reductase isolated from *E. coli* (Fischer *et al.*, 1990)

appears to be closely related to the ferric-iron reductase involved in ribonucleotide reduction and is recognised as a NAD(P)H:flavin oxidoreductase (Fontecave *et al.*, 1987). Finally, the flavin-containing NADH-nitrate reductase from squash cotyledon is capable of ferrisiderophore-reductase activity (Smarrelli and Castignetti, 1986) which futher demonstrates the lack of specificity of these enzyme systems.

All of these similarities suggest that the multi-step reduction involving a NADH/FMN reductase followed by FMN-mediated chemical reduction of the ferrisiderophore, may be a general mechanism occuring in all siderophore producing bacteria and fungi. Provided the inositol phosphate has access to the reductase system, it does appear that this system is capable of reducing inositol phosphate-bound Fe(III). This supports the hypothesis that bacterial reductase sytems are relatively non-specific.

Chapters 4,5 and 6 have demonstrated the ability of inositol phosphates to mediate iron transport and have identified several key trends. It has been possible to determine structure-iron transport relationships, how the ability to mediate iron transport is affected by their abilities to interact with iron and how this iron may be released to the bacterium. Chapter 7 will discuss the mechanisms whereby the bacterium is capable of using inositol phosphates as a means of iron acquisition.

Chapter 7

Assessing the Mechanism of Inositol Phosphate-Mediated Iron Transport in *Pseudomonas aeruginosa*

7.1. Introduction

The aim of this chapter is to explore the bacterial mechanisms which may be involved in inositol phosphate-mediated iron transport in *P. aeruginosa* PAO1. It examines the possible involvement of active transport mechanisms by studying the effects of low temperature and the presence of metabolic inhibitors. A series of experiments was performed to determine the role of the outer membrane and its associated proteins. Iron transport assays were performed using several strains that were deficient in the expression of several individual outer membrane proteins and in spheroplasts of *P. aeruginosa* PAO1 which lack the outer membrane. To characterise the effects of the outer membrane further, inositol phosphate binding assays were performed using *P. aeruginosa* outer membranes. Finally, attempts were made to isolate a transport-deficient mutant of *P. aeruginosa* unable to utilise *myo*-$InsP_6$ as an exogenous siderophore.

7.2 Assessing The Role of Active Transport Mechanisms.

The two parameters used to assess whether inositol phosphate-mediated iron transport in *P. aeruginosa* PAO1 was dependent on active transport were low temperature and the presence of a metabolic inhibitor. The compounds *myo*-$InsP_6$, *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,4,5)P_3 were chosen as they represent varying molecular weights. *myo*-$InsP_6$, molecular weight 923 Da, is regarded as too large to traverse porins *via* simple diffusion (Hancock *et al.*, 1979), whereas *myo*-Ins(1,4,5)P_3, with a molecular weight of 552 Da, may be capable of traversing porins in the outer envelope.

7.2.1. The Effect of Temperature on *myo*-Inositol Hexakisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa*

The effect of low temperature was assessed by removing a sample from the iron-transport reaction mixture at t=0.5 min and placing on ice for the duration of the assay. After 30 min, the chilled mixture was filtered and washed in the same manner as all other iron-transport assays. The cell-associated activity remaining on the filter was determined using β-scintillation counting.

Inositol Phosphate	Accumulated ^{55}Fe After 30 min at 4°C (pmol/10^9 cells) (n=1)	Accumulated ^{55}Fe After 30 min at 37°C (pmol/10^9 cells) (Mean±SEM, n=3)
myo-$InsP_6$	4.40	19.08±1.59
myo-Ins(1,2,4,5)P_4	53.34	95.83±19.93
myo-Ins(1,4,5)P_3	26.88	106.37±6.57

Table 7.1. The effect of low temperature on the ability of *myo*-$InsP_6$, *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,4,5)P_3 to mediate iron transport into *P. aeruginosa* PAO1 after 30 min.

Table 7.1. illustrates that temperature has a notable effect on the ability of the above inositol phosphates to mediate iron-transport. In all three cases, inositol phosphate-mediated iron transport was reduced considerably when performed at 4°C indicating that some component of the pathway is temperature dependent.

7.2.2. The Effect of Carbonyl Cyanide *m*-Chlorophenylhydrazone on *myo*-Inositol Hexakisphosphate-Mediated Iron-Transport in *Pseudomonas aeruginosa*

To establish whether active processes are required to facilitate inositol phosphate-mediated iron-uptake, transport assays were performed in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). CCCP uncouples oxidative reactions within the bacterial cell and consequently inhibits mechanisms requiring active energy. Assays were performed by addition of CCCP (1 mM) to the cell mixture

and incubated as usual prior to mixing with the chelate mixture at t=0 of the iron transport assay.

Inositol Phosphate	Accumulated ^{55}Fe After 30 min with Addition of CCCP (pmol/10^9 cells) (n=1)	Accumulated ^{55}Fe After 30 min (without CCCP) (pmol/10^9cells) (Mean±SEM, n=3)
myo-$InsP_6$	4.16	19.08±1.59
myo-Ins(1,2,4,5)P_4	32.23	95.83±19.93
myo-Ins(1,4,5)P_3	30.07	106.37±6.57

Table 7.2. The effect of CCCP on the ability of *myo*-$InsP_6$, *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,4,5)P_3 to mediate iron transport into *P. aeruginosa* PAO1 after 30 min.

Table 7.2. illustrates that addition of CCCP to the uptake medium resulted in a marked reduction in inositol phosphate-mediated iron transport into *P. aeruginosa* PAO1. This is consistent with the low temperature data and suggests that at least some component of inositol phosphate-mediated iron transport is dependent upon active processes.

7.3. The Role of the Outer Membrane in *myo*-Inositol Hexakisphosphate-Mediated Iron Transport in *Pseudomonas aeruginosa* PAO1

The role of the outer membrane in iron acquisition by *P. aeruginosa* PAO1 is well documented. Specific receptors for pyoverdine, pyochelin and enterobactin have been identified in the outer membrane (sections 1.4.2; 1.4.3; 1.7). In addition, several OMPs of *P. aeruginosa* still have no function assigned to them and there is

the possibility that one may be responsible for inositol phosphate-mediated iron uptake.

Iron transport assays were performed using strains of *P. aeruginosa* lacking either OprD, OprF or OprP. In addition, iron transport assays were performed in spheroplasts of *P. aeruginosa* which lack the outer membrane. Finally, a series of displacement binding assays were performed to determine the existence of specific binding sites.

7.3.1 *myo*-Inositol Hexakisphosphate-Mediated Iron-Transport in Strains of *Pseudomonas aeruginosa* Lacking Individual Outer Membrane Proteins.

myo-$InsP_6$-mediated iron transport was assayed in *P. aeruginosa* H729, lacking OprD expression, *P. aeruginosa* H636, lacking OprF expression and *P. aeruginosa* H576 which lacks expression of OprP. The OMP compositions of these strains are illustrated in figure 7.1. (p152). Figure 7.2. (p153) illustrates that each of these strains is still capable of undergoing *myo*-$InsP_6$-mediated iron transport indicating that none of these proteins are essential for *myo*-$InsP_6$-mediated iron transport.

7.3.2. Inositol Phosphate-Mediated Iron Transport in Spheroplasts of *Pseudomonas aeruginosa*

Spheroplasts of *P. aeruginosa* were prepared by treatment of log-phase cells with lysozyme and EDTA as described in section 3.8. Spheroplasts, which are osmotically sensitive, lack most of the outer membrane and peptidoglycan of the cell

wall and are useful in determining whether the outer layers of the cell wall play any role in inositol phosphate-mediated iron transport.

myo-$InsP_6$, *myo*-Ins(1,2,4,5)P_4, 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 and *myo*-Ins(1,4,5)P_3-mediated iron transport is illustrated by Fig 7.3. (p154). It is important to note that the x-axis represents iron uptake from 10^5 cells as determined by viable counting of a cell suspension of OD_{470} 1.0. In previous assays, iron uptake was expressed for 10^9 cells hence the actual amount of iron associated with each individual cell appears to be considerably greater when using spheroplasts.

It is interesting to note that all four compounds produced very similar iron transport profiles, with respect to both initial rate and accumulated values after 30 min. In spheroplasts, there appears to be a loss of all structure activity relationships. Even 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4, a compound associated with poor transport in normal cells, produced a transport profile similar to the other compounds.

Unfortunately, it was not possible to test the role of either low temperature or metabolic inhibitors as both of these caused lysis of the highly sensitive spheroplasts.

7.3.3. Competitive Binding Assays of Tritiated Inositol Phosphates to *Pseudomonas aeruginosa* Membranes.

Competitive binding assays were performed to assess whether *myo*-$InsP_6$ is capable of binding specifically to components of *P. aeruginosa* membranes in a manner indicative of the presence of specific receptors. For the data represented below,

binding was performed using outer membranes purified by the Sarkosyl method. Preliminary experiments were performed using *P. aeruginosa* total membrane fractions and outer membranes prepared using sucrose gradient purification; all gave similar binding results. Sarkosyl-purified outer membranes were preferred because of their relative ease of production compared to the sucrose gradient method. In addition, Sarkosyl-purified outer membranes were regarded to be more relevant to bacterial transport across the outer membrane than simply using total membrane fraction.

7.3.3.1 Competitive Binding Assays of [^{3}H]-InsP$_6$ to *Pseudomonas aeruginosa* Membranes.

A previous study by Smith *et al.*, (1994) using crude membrane fraction suggested the presence of specific *myo*-InsP$_6$ binding sites using self displacement of [^{3}H]-InsP$_6$ (K_d 143 nM) The aim of this study was to demonstrate binding to the outer membrane fraction and to determine whether displacement by a series of other compounds was possible. Competitive binding assays of [^{3}H]-InsP$_6$ to various *P. aeruginosa* membranes were performed as described in section 3.12. Assays measuring competition with [^{3}H]-InsP$_6$ binding by unlabelled *myo*-InsP$_6$, *myo*-Ins(1,2,4,5)P$_4$, 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P$_4$ and *myo*-Ins(1,4,5)P$_3$ were performed. *myo*-Ins(1,2,3)P$_3$ was also included to determine whether the 1,2,3-trisphosphate arrangement played any role in binding. Specific binding was only recorded with *myo*-InsP$_6$ (K_d 1200 nM) as illustrated in Fig. 7.4 (p155), all other compounds failed to displace [^{3}H]-InsP$_6$ over the concentration of unlabelled ligand

used in this assay. Table 7.3 illustrates the % dpm specifically bound at 100 μM (10,000 nM) using the above unlabelled compounds.

Unlabelled Inositol Phosphate	% [^{3}H]-InsP$_6$ Specifically Bound at 100 μM unlabelled ligand (Mean±SEM n=3)
myo-InsP$_6$	29±4
myo-Ins(1,2,4,5)P$_4$	98±1
3,6 di-O-benzoyl *myo*-Ins(1,2,4,5)P$_4$	99±1
myo-Ins(1,2,3)P$_3$	115±7
myo-Ins(1,4,5)P$_3$	80±7

Table 7.3. Competitive binding of 1 nM [^{3}H]-InsP$_6$ with unlabelled *myo*-InsP$_6$, *myo*-Ins(1,2,4,5)P$_4$, 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P$_4$, *myo*-Ins(1,2,3)P$_3$ and *myo*-Ins(1,4,5)P$_3$ (100μM) to sarkosyl-prepared outer membranes of *P. aeruginosa* PAO1 grown in succinate medium.

7.3.3.2 Competitive Binding Assay of [^{3}H]-InsP(1,4,5)$_3$ to *Pseudomonas aeruginosa* Outer Membranes with Unlabelled *myo*-Ins(1,4,5)P$_3$.

Throughout this study, *myo*-Ins(1,4,5)P$_3$ has appeared to be a particularly good mediator of iron transport in *P. aeruginosa* PAO1 which raised the possibility of specific *myo*-Ins(1,4,5)P$_3$ binding to the bacterial cell surface. One preliminary binding assay was performed to determine the ability of *myo*-Ins(1,4,5)P$_3$ to compete with the binding of [^{3}H]-InsP(1,4,5)$_3$. Because [^{3}H]-InsP(1,4,5)$_3$ was available in a very limited quantity, only one assay was performed. However, from the results obtained, there was no apparent specific binding. The results are illustrated in Table 7.4.

Unlabelled *myo*-Ins(1,4,5)P_3 (nM)	% [^{3}H]-Ins(1,4,5)P_3 Bound (Mean ± SEM, n=3)
1	92±16
10	70±5
100	72±3
1000	70±2
10000	72±6

Table 7.4. Competitive binding of 1 nM [^{3}H]-Ins(1,4,5)P_3 with unlabelled *myo*-Ins(1,4,5)P_3 to sarkosyl-prepared outer membranes of *P. aeruginosa* PAO1 grown in succinate medium.

7.4. Transposon Insertion Mutagenesis of *Pseudomonas aeruginosa*

This chapter demonstrated the importance of active transport mechanisms and has identified the outer membrane as a key factor in *myo*-InsP_6-mediated iron transport. However, the exact mechanism of action is unknown. In order to assign a mechanism of action of these compounds, attempts were made, using transposon mutagenesis, to obtain a mutant unable to use ferri-*myo*-InsP_6 as a source of iron. Two strains of *P. aeruginosa* were used which were deficient in endogenous siderophore production but were still capable of undergoing *myo*-InsP_6-mediated iron transport.

A derivative of *P. aeruginosa* IA1, PH3, was mutagenised using Tn*501* in plasmid pMT1000. Secondly, a derivative of *P. aeruginosa* K372, PH4, was mutagenised using Tn*1737*KH contained in plasmid pMT6121. It was decided not to use the Tn*5*-based system contained in pUW964 as Tn*5*-based mutations have been found to be

unstable in *P. aeruginosa* (Cornelis *et al.*, 1992 and our own observations with *P. aeruginosa* PH1, Smith *et al.*, 1992). Both *P. aeruginosa* IA1 and *P. aeruginosa* K372 were capable of *myo*-$InsP_6$-mediated iron transport (Figure 7.5 - p156) and the outer membrane protein compositions of these strains are illustrated in figure 7.1.

7.4.1. Tn*501* Mutagenesis of *Pseudomonas aeruginosa* PH3

Tn*501*, a transposon encoding mercury resistance, was used in insertion mutagenesis using pMT1000, a temperature-sensitive derivative of plasmid R68, (Tsuda *et al.*, 1984). Tn*501* is bound by small inverted repeats and is capable of transposing into a wide variety of hosts (Bennett *et al.*, 1978). pMT1000 was conjugated into *P. aeruginosa* IA1 to produce *P. aeruginosa* PH3 as described previously (section 3.14.1.2).

At the restrictive temperature of 42°C, the frequency of mercury resistant cells was $1.6x10^{-4}$/viable cell. This is similar to the value obtained by Tsuda *et al.*, (1984) when mutagenising *P. aeruginosa* PAO1 with pMT1000 (they reported $4.8x10^{-4}$). In addition to the Tn*501*-encoded mercury resistance associated with pMT1000, the plasmid also encodes tetracycline resistance that is not associated with Tn*501*. Consequently, 100 mercury resistant transformants were tested for tetracycline resistance and all were found sensitive. This confirmed that the vector was lost at the restrictive temperature and the mercury resistance was a result of transposition of Tn*501* into the chromosome.

Transposition of *P. aeruginosa* was performed and 25,000 colonies were screened on succinate minimal agar, succinate minimal agar + 500 μM $InsP_6$, Luria agar + 500 μ M $InsP_6$ and Luria agar + Hg^{15} as described in section 3.14.1.4.

Approximately 5% of transposed colonies were unable to grow on minimal media yet were able to grow on complex media, illustrating that the system was producing nutritional auxotrophs. However, despite these efforts, no strains unable to use *myo*-$InsP_6$ as an iron source were isolated.

7.4.2. Tn*1737*KH Mutagenesis of *Pseudomonas aeruginosa* PH4

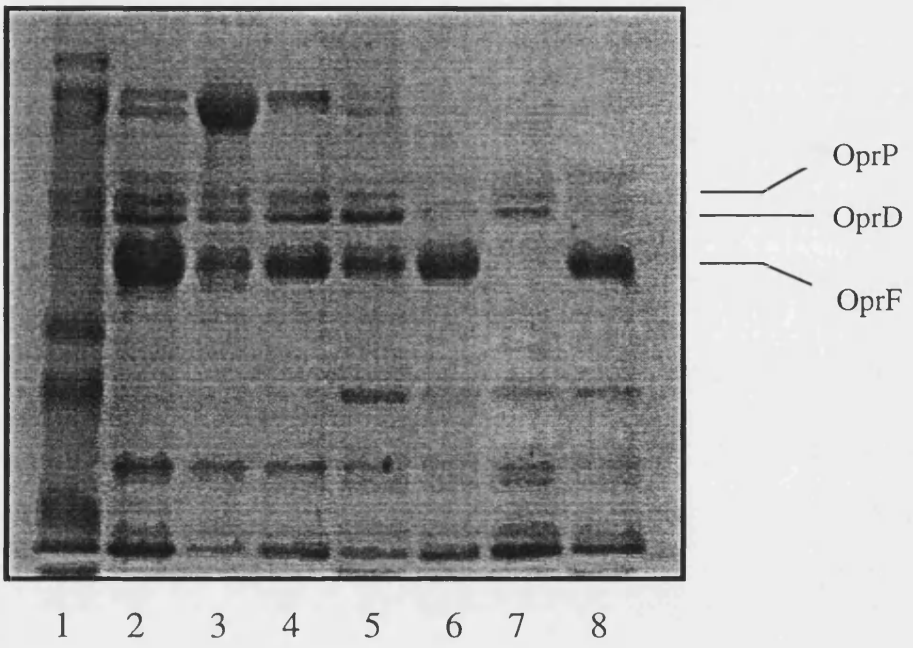
Tn*1737*KH on plasmid pMT6121 was conjugated into *P. aeruginosa* K372 to produce *P. aeruginosa* PH4. Tn*1737*KH is a combination of Tn*1737*Km (Ubben and Schmitt, 1987), which encodes kanamycin resistance, and a mercury-resistant element. The mercury-resistant element, a fragment from pHP45ΩHg, was introduced because *P. aeruginosa* is intrinsically resistant to high concentrations of kanamycin (Tsuda *et al.*, 1995). Plasmid pMT6121 is a derivative of plasmid R68 which is temperature sensitive and encodes tetracycline resistance.

Again, the frequency of transformation of *P. aeruginosa* PH4(pMT6121::Tn*1737*KH) was calculated. At the restrictive temperature of 42°C, the frequency of mercury resistant cells was and found to be $1.5x10^{-5}$/viable cell which is again similar to $8x10^{-5}$ reported by Tsuda *et al.*, (1995). One hundred transposed cells grown at the restrictive temperature and encoding mercury resistance were screened for tetracycline sensitivity. All colonies tested were tetracycline

sensitive indicating loss of the vector and that mercury resistance was a result of Tn*1737*KH incorporation into the chromosome.

Transposon mutagenesis of *P. aeruginosa* PH4 was performed and 25,000 colonies screened on succinate minimal agar + 1 mM Met, succinate minimal agar + 1 mM Met + 500 μM $InsP_6$, Luria agar + 500 μM $InsP_6$ and Luria agar + Hg^{15} as described in section 3.13.2.2. Growth on selective media indicated that 5-10% of cells were nutritional auxotrophs confirming that the mutagenesis system was working well. However, it was never possible to isolate a mutant unable to use *myo*-$InsP_6$ as a source of iron.

Figure 7.1. SDS-PAGE of *P. aeruginosa* Outer Membrane Proteins.



Lane 1	Bio-Rad Molecular Weight Markers
Lane 2	*P. aeruginosa* PAO1 (Succinate Minimal Medium)
Lane 3	*P. aeruginosa* K372 (Succinate Minimal Medium)
Lane 4	*P. aeruginosa* IA1 (Succinate Minimal Medium)
Lane 5	*P. aeruginosa* PAO1 (Luria Broth + 400 μM EDDHA)
Lane 6	*P. aeruginosa* H729, OprD- (Luria Broth + 400 μM EDDHA)
Lane 7	*P. aeruginosa* H636, OprF- (Luria Broth + 400 μM EDDHA)
Lane 8	*P. aeruginosa* H576, OprP- (Luria Broth + 400 μM EDDHA)

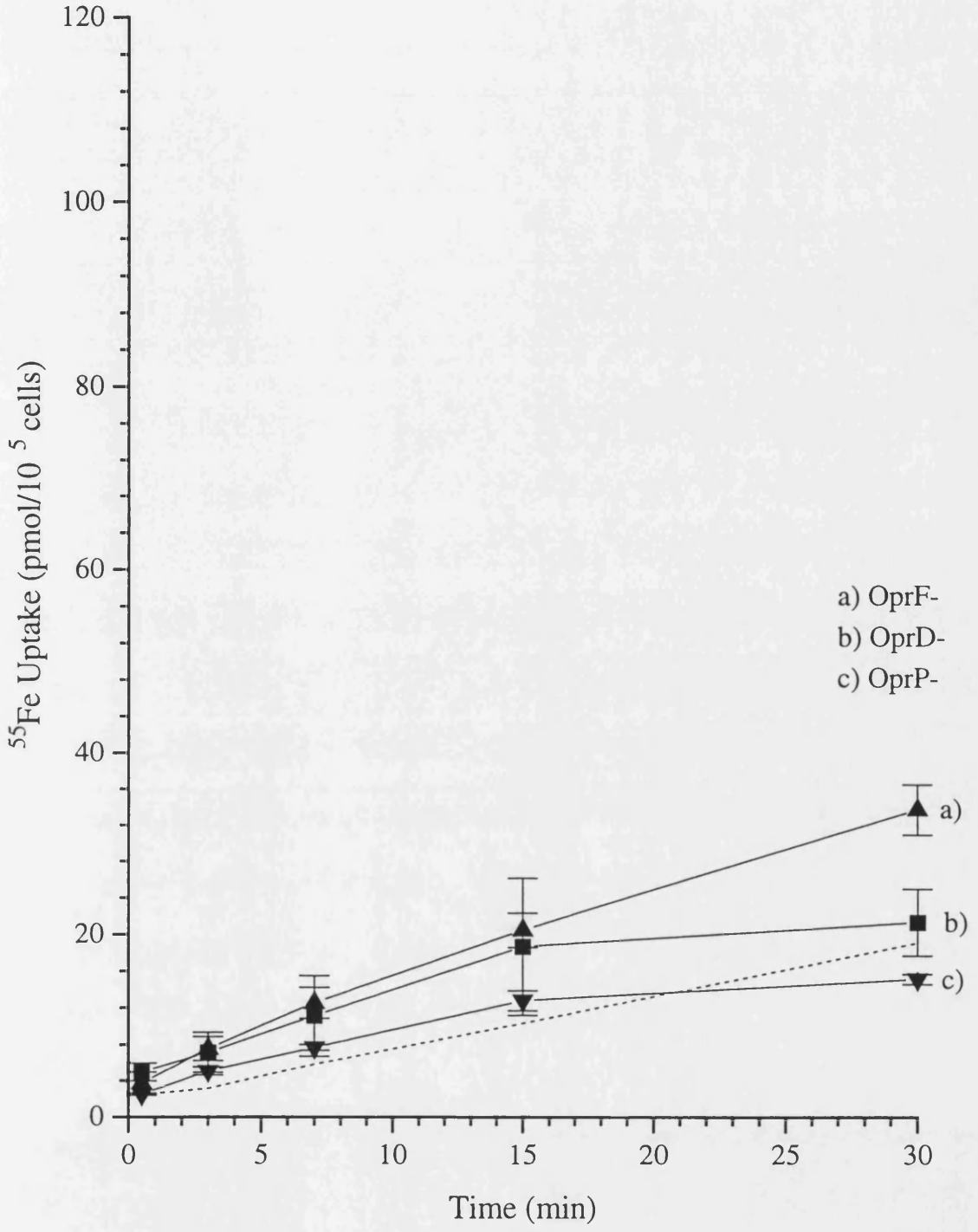


Figure 7.2. *myo*-$InsP_6$-mediated iron transport in strains of *P. aeruginosa* lacking individual outer membrane proteins grown in LB + EDDHA (400 μM). The uptake media contained *myo*-$InsP_6$ (100 mM), $^{55}FeCl_3$, glucose (60 μM) and 1 ml of cells OD_{470} 1.0. The dashed line represents *myo*-$InsP_6$-mediated iron transport into *P. aeruginosa* PAO1.

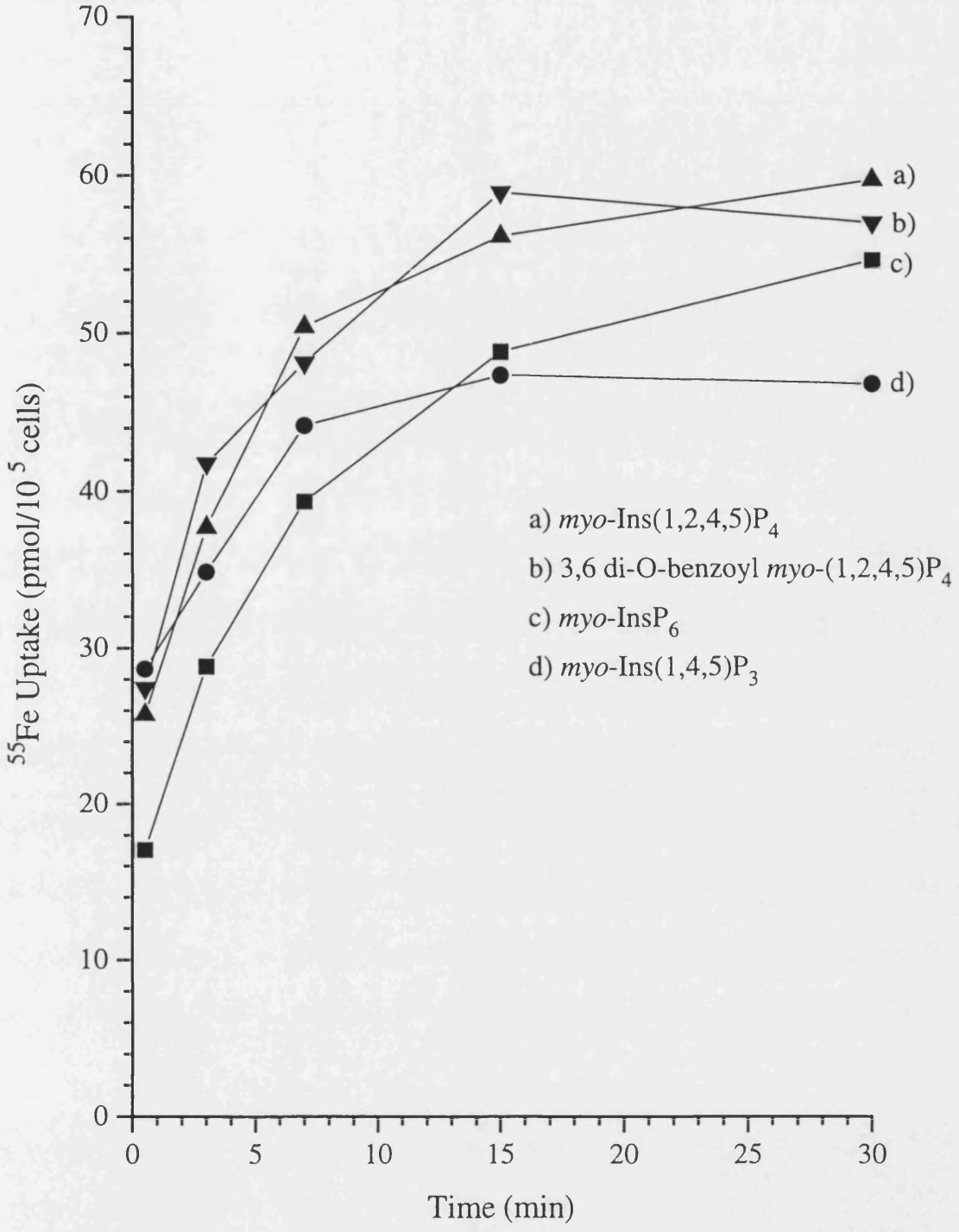


Figure 7.3 Inositol phosphate-mediated iron transport in *P. aeruginosa* PAO1 spheroplasts grown in succinate medium. The uptake media contained inositol phosphate (100 μM), $^{55}FeCl_3$ (200 nM) and 1 ml of spheroplasts OD_{470} 1.0. (10^5 cells). N.B. The data represent n=3 and error bars have been omitted for clarity.

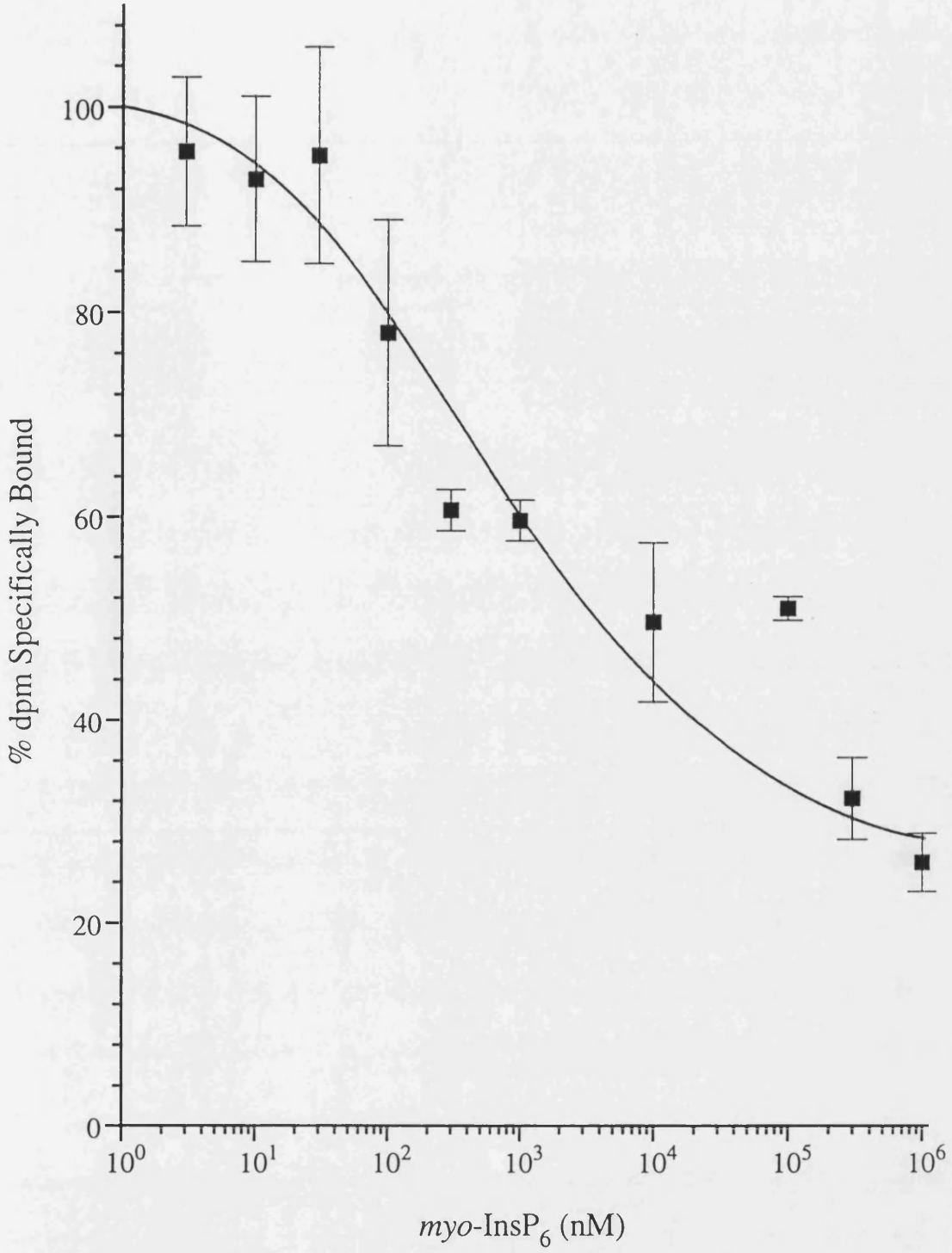


Figure 7.4 Competitive binding of 1 nM ^{3}H-$InsP_6$ with unlabelled *myo*-$InsP_6$. Each binding mixture contained 0.5 mg sarkosyl-prepared outer membrane from succinate grown *P. aeruginosa* PAO1.

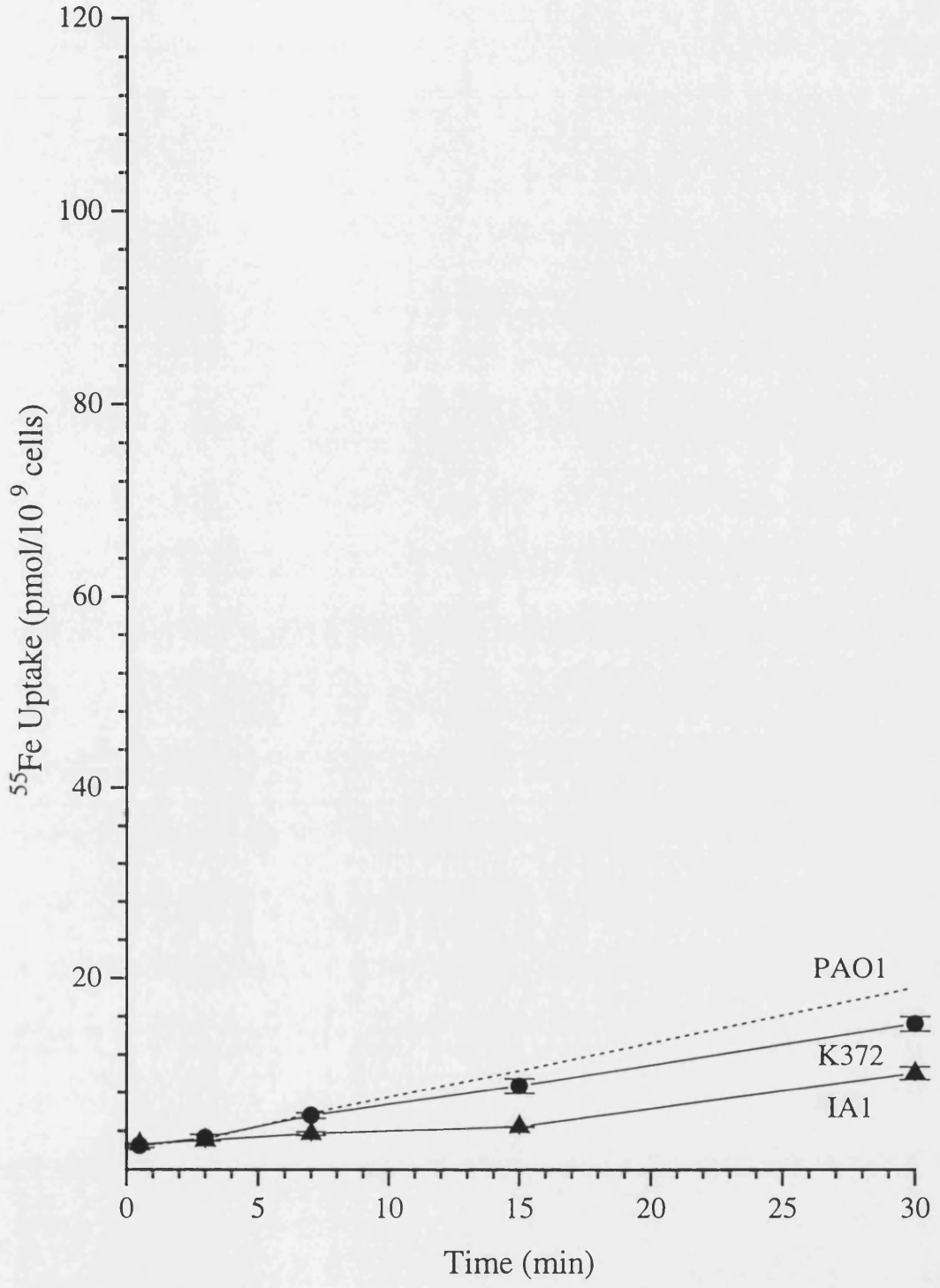


Figure. 7.5. *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* IA1 and *P. aeruginosa* K372 grown in succinate medium. The uptake media contained *myo*-$InsP_6$ (100mM), $^{55}FeCl_3$ (200nM) and 1.0. ml of cells OD_{470} 1.0. The dashed line is duplicated from Fig. 4.2. for comparison.

7.5. Discussion

The chapter has achieved several goals in attempting to elucidate the mechanisms by which *P. aeruginosa* PAO1 can utilise *myo*-$InsP_6$ as a means of iron acquisition. However, it was not possible to isolate a transport mutant.

In particular, this chapter has highlighted the importance of the Gram-negative outer membrane. It was of interest that all the Opr-deficient mutants tested were able to undergo *myo*-$InsP_6$-mediated iron uptake. Under normal conditions, OprD is thought to be responsible for the uptake of basic amino acids and has also been implicated in the uptake of imipenem (Yoshihara and Nakae 1989; Quinn *et al.*, 1986; Trias *et al.*, 1989). Meyer (1992), noted that strains lacking OprD were deficient in desferriferrioxamine E-mediated iron transport hence suggesting a role for this porin in iron transport. However, it is apparent that this protein is not involved with *myo*-$InsP_6$-mediated iron transport. The exact role of OprF is unclear, although it appears to be associated with general porin activity and cell structure (Woodruff *et al.*, 1986; Gotoh *et al.*, 1989; Woodruff and Hancock, 1988). OprF is of particular interest because of its previous association with exogenous siderophore-mediated iron transport into *P. aeruginosa* (Meyer, 1992). In comparison to the parent strain, *P. aeruginosa* PAO1, the OprF-deficient mutant was unable to use desferriferrioxamine E, desferriferrioxamine B, desferriferricrocin and desferriferrichrysin. However, in this study, *myo*-$InsP_6$-mediated iron transport was unaffected in the OprF-deficient mutant. The association of OprP with phosphate transport in *P. aeruginosa* (Hancock *et al.*, 1982; Worobec *et al.*, 1988; Poole and Hancock, 1986) makes an OprP deficient strain particularly interesting with respect to inositol phosphate-

mediated iron transport The rationale for its use was whether the phosphate moieties of *myo*-$InsP_6$ would have affinity towards this particular OMP even though maximal expression occurs in phosphate deplete conditions which are not encountered in succinate minimal medium. However, there was little difference between this and the parent strain in their abilities to undergo *myo*-$InsP_6$-mediated iron transport. In addition, the siderophore-deficient strains *P. aeruginosa* IA1 and K372 were both able to undergo *myo*-$InsP_6$-mediated iron transport despite lacking components of the outer membrane associated with ferrisiderophore uptake as illustrated in figure 7.1.

The importance of the outer membrane was particularly apparent when performing iron transport assays in spheroplasts of *P. aeruginosa* PAO1. The iron uptake profiles associated with various inositol phosphates were greater than those achieved using normal cells. This indicates that relatively large amounts of iron become associated with these delicate cells. Also of interest was the total lack of structure-activity relationships normally associated with these compounds which may represent differences in the way individual inositol phosphates present iron to the altered bacterial cell surface.

The ability of *myo*-$InsP_6$ to interact with the outer membrane was illustrated by competitive binding experiments using [^{3}H]-$InsP_6$. The data indicated competitive binding between [^{3}H]-$InsP_6$ and *myo*-$InsP_6$ which was in agreement with previous work (Smith *et al.*, 1994). However, none of the other compounds tested were able to displace [^{3}H]-$InsP_6$ from the outer membranes suggesting that the binding was specific to *myo*-$InsP_6$. In addition, considerable attempts were made to demonstrate

specific [^{3}H]-InsP_6 binding to whole cells of *P. aeruginosa* although binding appeared too transient to be recorded (data not shown). Also, attempts were made to separate outer membrane fractions using column chromatography to determine whether particular membrane fractions were responsible for [^{3}H]-InsP_6 binding. However, difficulties achieving complete solubilisation of outer membranes without impairing column efficiency prevented these studies (data not shown). Because of the observations that *myo*-Ins(1,4,5)P_3 was the most efficient mediator of iron transport in this study, a preliminary experiment was performed to determine whether this compound was capable of binding specifically to the outer membrane. However, no specific binding was observed.

Several studies have examined the ability of *myo*-InsP_6 to bind specifically to various eukaryotic sites. Nicoletti *et al.*, (1990) noted the presence of *myo*-InsP_6 receptors at various sites within the brain including cerebral hemispheres, anterior pituitaries and cerebellar neurones. Interestingly, they noted that specifically bound [^{3}H]-InsP_6 was not displaced by *myo*-Ins(1,4,5)P_3, a phenomenon also noted in this study. Hawkins *et al.*, (1990) noted *myo*-InsP_6 binding sites on neuronal cell bodies which was distinct from previously characterised Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 binding. These data suggested a possible extracellular role as a neuronal excitant. In support of this role, Theibert *et al.*, (1991) suggested that the *myo*-InsP_6 receptor also appeared to affect calcium accumulation and the locations of the receptors were consistent with a neuronal, synaptic role. Later work by Voglemaier *et al.*, (1992) and Theibert *et al.*, (1992) suggested that the *myo*-InsP_6 receptor isolated from bovine cerebellar membranes is essentially identical to the clathrin assembly protein AP-2. AP-2

promotes assembly of clathrin into coated vesicles at the plasma membrane and is proposed to be involved with receptor recycling. The proposed links between the *myo*-$InsP_6$ receptor and AP-2 suggest that *myo*-$InsP_6$ has roles both in signalling pathways and receptor-mediated endocytosis Voglemaier *et al.*, (1992).

Of particular interest to this study was the observation that [^{3}H]-$InsP_6$ would bind specifically to cerebellar membranes (Poyner *et al.*, 1993) possibly *via* phospholipids. One suggestion for this mechanism was that positively charged cations form a bridge between the negatively charged phospholipid and the negatively charged *myo*-$InsP_6$. It is thought that the inositol phosphate may not have sufficiently high affinity for the metal ion to remove it from the phospholipid. With respect to *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa*, this is particularly important with respect to the possible bridging effect between the inositol phosphate, Fe(III) and the negatively charged phospholipids of the bacterial outer membrane. The effect of such bridging mechanisms on the outer membrane is unknown.

It is interesting to note that inositol phosphate-mediated iron transport depends upon active transport systems with both low temperature and CCCP having negative effects. In general, low temperature and CCCP reduced iron transport to approximately one third of that normally seen after 30 minutes. This is particularly interesting with respect to *myo*-Ins(1,4,5)P_3 as the relatively low molecular weight of this compound (552 Da) is within the size range of compounds capable of entering the bacterium *via* porin-mediated passive diffusion. In general, hydrophilic compounds up to 600 Da may permeate from the growth medium to the periplasm

(Nikaido and Saier, 1992; Benz, 1994) although this is unlikely for highly charged inositol phosphates. Clearly, the enhanced iron transport normally associated with this compound is not a result of diffusion. It is also noteworthy that even in the presence of a metabolic inhibitor, the total amount of iron accumulated after 30 minutes was never zero. This may be a result of binding to the bacterial cell surface or the activity of non-specific, low affinity iron transport mechanisms.

The dependence upon active transport suggests the involvement of either a TonB-type system, a periplasmic-binding-protein-dependent system, or both. Much of the work regarding TonB-mediated active transport has centred around *E. coli* vitamin B_{12} transport. Vitamin B_{12} interacts with the OMP BtuB and is transported across the outer membrane resulting in 1000-fold concentration in the periplasm relative to the growth medium. The next stage is interaction with the periplasmic binding protein BtuC. Mutations in *btuC* result in inhibition of vitamin B_{12} transport across the cytoplasmic membrane although vitamin B_{12} will still concentrate in the periplasm (Reynolds *et al.*, 1980; Bradbeer, 1993). Consequently, active transport of this nutrient across the outer membrane is independent of periplasmic binding proteins. Vitamin B_{12} transport across the outer membrane is also blocked by CCCP suggesting that the energy source must be the cytoplasmic membrane (Postle, 1993). The ability of TonB to act as an energy transducer and the necessity for the cytoplasmic membrane electrochemical potential were confirmed by Jaskula *et al.*, (1994). It was shown that cleavage of TonB to separate the periplasmic domain from the cytoplasmic domain inhibited TonB function. Furthermore, they located specific sites within the cytoplasmic membrane domain that when removed, inhibited energy

transduction between the cytoplasmic and outer membranes. Clearly, vitamin B_{12} transport relies on active transport mechanisms whereby energy is supplied from the cytoplasmic membrane to the outer membrane *via* the action of TonB. It is therefore possible that *myo*-$InsP_6$-mediated iron transport is dependent upon such a system.

Alternatively, inhibition of *myo*-$InsP_6$-mediated iron transport by CCCP may be the result of inhibiting a periplasmic-binding-protein-dependent system. These systems have been identified in the iron uptake systems of several species of bacteria including *E. coli* (Stephens *et al.*, 1995), *Serratia marcescens* (Angerer *et al.*, 1992), *Haemophilus influenzae* (Adhikari *et al.*, 1995) and *Neisseria gonorrhoeae* (Adhikari *et al.*, 1996). The roles of the FepABCDG proteins in enterobactin-mediated iron transport in *E. coli* are well documented as indicated in the introduction (section 1.6.2; Stephens *et al.*, 1995). In this system, the periplasmic binding protein, FepB, recognises the ferrienterobactin complex. For *H. influenzae* and *N. gonorrhoeae*, the periplasmic binding protein recognises free ferric iron released from the outer membrane receptors. However, for *S. marcescens*, the mechanism by which iron traverses the outer membrane is unknown although SfuA, the periplasmic binding protein of *S. marcescens*, will accept iron solubilised with oxaloacetate, sodium PP_i and citrate. There is distinct homology between the *sfuABC*, *hitABC* and *fbpABC* loci of *S. marcescens*, *H. influenzae* and *N. gonorrhoeae*, respectively. Briefly, each system comprises a ferric binding protein (SfuA/HitA/FbpA), a hydrophobic cytoplasmic permease (SfuB/HitB/FbpB) and a nucleotide binding protein (SfuC/HitC/FbpC). The nucleotide binding protein facilitates hydrolysis of a

nucleotide phosphate, e.g. ATP which provides energy for this uptake system. This system is termed an ABC (ATP Binding Cassette) transporter-exporter system.

The observation that *myo*-$InsP_6$ was not able to induce any detectable change in OMP composition of *P. aeruginosa* PAO1 (Smith *et al.*, 1994) suggests that this system is unlikely to depend upon TonB-dependent receptor systems. Similarly, iron-uptake assays using strains deficient in individual OMPs and siderophore production, and the lack of success using transposon mutagensis systems all support this theory It is therefore possible that this system relies upon a periplasmic-binding-protein-dependent system analogous to the *sfu* system of *S. marcescens*. Whilst the *hit* and *fbp* systems of *H. influenzae* and *N. gonorrhoeae* bind free ferric iron released into the periplasm following receptor recognition of ferri-glycoprotein complexes, the *sfu* system of *S. marcescens* appears most analogous to *myo*-$InsP_6$-mediated iron transport in light of the lack of an associated outer membrane protein.

When the *sfuABC* locus was inserted into *E. coli*, expression of *sfuABC* was dependent upon Fur activity (Angerer *et al.*, 1992). Parallels can be drawn with *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* which is iron-regulated (Smith *et al.*, 1994) and may also involve the Fur protein. Using *E. coli sfuABC* transformants lacking some of the usual components of siderophore-mediated iron transport, it was noted that not all siderophores could be transported across the cytoplasmic membrane by the Sfu system. These included ferrichrome, coprogen and dihydroxybenzoate. In addition, studies were performed to examine citrate mediated-iron transport in *sfuABC* transformants of *E. coli* mutated in the *fecBCDE*

genes. Under moderate iron limitation, citrate would carry iron across the outer membrane and the Sfu system would mediate iron transport across the cytoplasmic membrane in sufficient quantities to permit growth. However, in conditions of severe iron deficiency, citrate transport required the presence of the FecA receptor and an intact TonB system. This may be because under conditions of moderate iron depletion, the iron diffusion rate is sufficiently high to fulfill the iron requirement whereas when iron levels are particularly low, active energy, as supplied by TonB, is required to facilitate transport across the outer membrane. The probable reason for the inability of ferrichrome, coprogen and dihydroxybenzoate to serve as iron donors to the Sfu system may be because their iron affinities are too high. Citrate however, probably binds iron with sufficient affinity that a transport system has evolved for this carrier and yet sufficiently weak to donate it to the Sfu system. Using *E. coli sfuABC* transformants it was noted that citrate-mediated iron transport *via* the Sfu system was considerably more efficient than *via* the Fec system, further illustrating the success with which citrate functions as an iron transporter (Angerer *et al.*, 1992). Using a series of strains of *S. marcescens*, Angerer *et al.*, (1992) demonstrated the existence of ferrienterobactin, ferriaerobactin, ferrichrome, ferrioxamine B, ferricitrate and hemin transport proteins although enterobactin was the only siderophore synthesised by the strains tested. Interestingly, despite the ability of citrate to act as a mediator of iron transport in *S. marcescens*, Angerer *et al.*, (1992) were unable to induce the expression of a citrate receptor in the outer membrane. Consequently, the exact mechanism of transport of the ferricitrate complex across outer membrane remains unclear. This is analogous with *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* in which it was not possible to induce a specific outer

membrane receptor. If such a system does exist in *P. aeruginosa*, *myo*-$InsP_6$, like citrate, may be a suitable donor of ferric iron to a periplasmic binding protein.

The final aspect of this study was the attempt to create a mutant strain that was unable to undergo *myo*-$InsP_6$-mediated iron transport. Despite the screening of approximately 50,000 mutants, none were unable to use iron complexed with *myo*-$InsP_6$. The estimation that a bacterial chromosome comprises approximately 10,000 genes indicates that the scale of the screening procedure was sufficient. However, other workers have experienced problems when trying to isolate iron transport-deficient mutants of *P. aeruginosa*. Iron transport studies involving the endogenous siderophore pyoverdine noted that a mutant deficient in production of the 90 kDa ferripyoverdine receptor, FpvA, still exhibited residual pyoverdine-mediated iron transport (Poole *et al.*, 1991). Poole *et al* (1991) suggested the presence of a second uptake system for ferripyoverdine which was not affected by high iron concentrations. Additionally, growth of the mutant in the presence of pyoverdine did not enhance this residual pyoverdine-dependent iron uptake indicating that if such a second system did exist, then it was not pyoverdine inducible. A similar phenomenon was observed with enterobactin-mediated iron uptake in *P. aeruginosa* (Poole *et al.*, 1990). Whilst a mutant was obtained that lacked the enterobactin-inducible 80 kDa PfeA outer membrane protein, it was still able to undergo enterobactin-mediated iron transport, albeit at a considerably reduced rate. Interestingly, this iron uptake was independent of whether the mutant had been grown in the presence of enterobactin or whether the growth medium contained high or low levels of iron. Even when the parent strain was cultured without enterobactin

and in the presence of high levels of iron, it was still capable of a limited amount of enterobactin-mediated iron uptake. This suggested the presence of two uptake mechanisms which would therefore require at least two mutations to eliminate them. It therefore appears that a single mutation in either the pyoverdine or enterobactin-mediated iron transport systems is insufficient to eliminate iron transport *via* that route. As suggested by Poole *et al.* (1990), "the potential for multiple uptake pathways for the same iron-siderophore complexes undoubtedly reflects the need to acquire this essential and not readily obtainable nutrient." The lack of success in obtaining a mutant unable to undergo *myo*-$InsP_6$-mediated iron transport may therefore be a result of more than one transport system being in operation.

Another possibility for the lack of success may be that the mutation required to prevent this uptake mechanism may be lethal to the bacterium, for instance by deletion of a key receptor, enzyme or structural component. There are several possibilities. One such possibility is that the only way to prevent *myo*-$InsP_6$-mediated transport is *via* the deletion of a global iron transport regulator such as the *fur* gene. Isolation of a *fur*-null mutant has not been possible, probably a result of lethality (Prince *et al.*, 1993; Ochsner *et al.*, 1995). Previous work in this chapter illustrated the importance of active processes in *myo*-$InsP_6$ mediated transport. If *myo*-$InsP_6$-mediated transport is *via* a periplasmic-binding-protein-dependent active transport system, then again, deletion of of such a system may be lethal. The work regarding reductase activity may provide an additional suggestion. It was noted that reductase activity was capable of removing Fe(III) from a variety of inositol phosphate compounds by reduction to Fe(II) and may play a key role in *myo*-$InsP_6$-mediated

iron transport. It is interesting to note that the ferrichrome reductase isolated from *E. coli* (Fischer *et al.*, 1990) appears to be closely related to the ferric-iron reductase involved in ribonucleotide reduction and is recognised as a NAD(P)H:flavin oxidoreductase (Fontecave *et al.*, 1987). It is therefore possible that *P. aeruginosa* reductase performs similar functions suggesting it may be an important homeostatic regulator rather than purely a siderophore-specific enzyme. Consequently, deletion of genes associated with this enzyme may also be lethal. Finally, the observation by Poyner *et al.*, (1993) that *myo*-$InsP_6$ binds specifically with membrane phospholipids may provide a further suggestion. It is possible that the inositol phosphate-bacterium interaction is *via* a cationic bridging mechanism and may be a key event in this transport mechanism. This may also be a reflection of the apparent inability of *myo*-$InsP_6$ to induce changes in the outer membrane protein composition of *P. aeruginosa* PAO1 as indicated by Smith *et al.*, (1994). If such a system is in operation, then a mutation in outer membrane phospholipid synthesis would be severely detrimental to the survival of the bacterium.

In summary, whilst this chapter was unable to identify an exact mechanism of action of *myo*-$InsP_6$-mediated iron transport, several key elements gave been identified. Briefly, a large component of this phenomenon is dependent upon active transport, and whilst the outer membrane plays a key role in this mechanism of transport, the involvement of a specific outer membrane protein cannot be demonstrated. The inability to isolate a mutant unable to use *myo*-$InsP_6$ as a source of iron may reflect the involvement of a key structural component, binding protein or enzyme system. A

model is proposed which indicates a periplasmic-protein-dependent system although the mechanism of transport across the outer membrane is unclear.

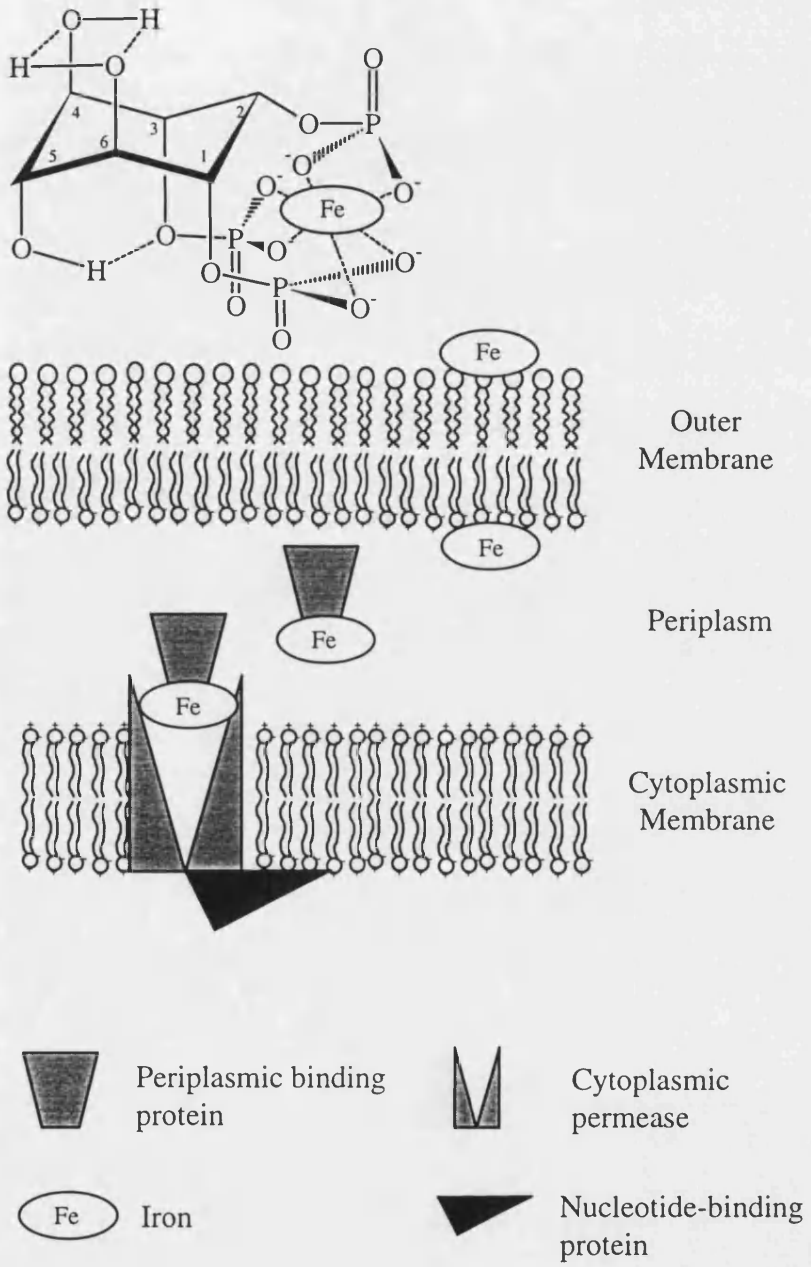


Figure 7.6. Model for inositol phosphate-mediated iron transport in *P. aeruginosa*. This diagrammatic representation illustrates *myo*-Ins(1,2,3)P_3 presenting iron to the surface of the bacterium with a periplasmic-binding-protein-dependent system responsible for transport across the cytoplasmic membrane.

Chapter 8

Concluding Remarks

Following the first report of *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* PAO1 by Smith *et al.*, (1994), this study has characterised the phenomenon further. Using a range of inositol phosphate compounds, it has been possible to determine several structure-iron transport activities and relate these differences to their relative abilities to interact with iron. This study also made some assessment of how these compounds yield iron to the bacterium and finally assesses the mechanisms by which the bacterium is able to accumulate iron using these compounds.

To determine the key structural elements responsible for *myo*-$InsP_6$-mediated iron transport, a series of iron transport assays was performed using a range of lower inositol phosphates. It was evident that these compounds produced a diverse range of iron uptake profiles and certain key trends became apparent. Most notable were the activities surrounding the 1,2,3 trisphosphate motif and the 1,5 bisphosphate motif. Briefly, those compounds possessing the 1,2,3 trisphosphate motif are poor mediators of iron transport whereas the 1,5-bisphosphate motif appears to be conducive to mediating iron transport in *P. aeruginosa*. To develop these observations further, the hydroxyl radical assay was used to determine the ability of these compounds to interact with Fe(III). The 1,2,3 trisphosphate motif appears particularly effective at

interacting with Fe(III) in such a manner that inhibits hydroxyl free radical generation. Such an interaction may, therefore, prevent the compound from liberating iron to a putative carrier system resulting in a low iron transport profile. In contrast, the 1,5-bisphosphate motif does not appear to be particularly effective in inhibiting Fe(III)-catalysed hydroxyl free radical formation. Consequently, the enhanced iron transport associated with this motif may reflect the ease with which iron is liberated to a putative carrier system. Competition iron transport studies indicated that the apparent differences in the affinities for iron are sufficient to permit competition for the metal. The inability of *myo*-$InsP_6$ to compete with a non-utilisable pyoverdine for available iron, indicated that inositol phosphate-mediated iron transport may not necessarily confer a competitive advantage.

It was also noted that the presence of large functional groups appears to reduce inositol phosphate-mediated iron transport. These groups made little difference to the ability of 3,6 di-*O*-benzoyl *myo*-Ins(1,2,4,5)P_4 to interact with Fe(III) despite conferring a low iron uptake profile. This suggests that the large sterically hindering groups reduce the ability of the compound to interact with the putative iron transport system.

In attempting to assess the mechanisms by which iron is released from the ferri-inositol phosphate complex, two key observations were made. Firstly, the use of a conformationally restricted compound indicated that the ability of inositol phosphates to undergo a change of ring conformation was not an essential component of *myo*-$InsP_6$-mediated iron transport. Secondly, the *P. aeruginosa* reductase system is

capable of reducing inositol phosphate-bound Fe(III) to Fe(II) with a subsequent release from the ferri-inositol phosphate complex. Whilst Halle and Meyer (1992a) noted that *P. aeruginosa* ferri-siderophore reductase activity was limited to the cytoplasm, this study does not indicate whether the ferri-inositol phosphate complexes are actually internalised.

Attempts to determine the mechanism by which inositol phosphates mediate iron transport into *P. aeruginosa* noted the importance of the outer membrane and the dependence upon active transport systems. Clearly, the outer membrane plays a key role as its removal led to considerably enhanced inositol phosphate-mediated iron transport and a loss of all structure-iron transport relationships. It was also possible to demonstrate specific binding to some component of the outer membrane although it was not possible to demonstrate the involvement of a specific outer membrane protein. The use of a metabolic inhibitor demonstrated that at least some component of inositol phosphate-mediated iron transport was dependent upon active systems. Finally, despite considerable attempts, it was not possible to isolate a mutant unable to use *myo*-$InsP_6$ as a source of iron.

In light of the evidence above, *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* may occur *via* a periplasmic-binding-protein-dependent system analogous to the Sfu system of *S. marcescens*. In support of this theory are the observations that both citrate-mediated iron transport in *S. marcescens* and *myo*-$InsP_6$-mediated iron transport in *P. aeruginosa* PAO1 are unlikely to depend upon a TonB-dependent OM receptor although they are both active processes. However, whilst the outer

membrane plays a key role in inositol phosphate-mediated iron transport in *P. aeruginosa* PAO1, the exact mechanism is unclear. It was demonstrated that *myo*-$InsP_6$ was capable of binding specifically to some component of the *P. aeruginosa* outer membrane, perhaps phospholipids. This mechanism may form the basis of the inositol phosphate-bacterial outer membrane interaction. It is unlikely that inositol phosphates would traverse the hydrophobic outer membrane due to their highly charged structures hence they may simply present iron to a putative carrier system at the bacterial surface. The apparent inverse relationship between the ability to mediate iron transport and the ability to interact with iron, may reflect the ease with which these compounds yield iron to such a carrier system. Such interactions would explain why the presence of large sterically hindering groups impair the inositol phosphate:bacterium interaction and why D- and L-enantiomers of several compounds mediated similar uptake profiles. The free ferric iron released to the periplasm may then interact with a periplasmic binding protein analogous to SfuA of *S. marcescens*. Consequently, removal of the outer membrane removes the first site of interaction exposing various components of the cytoplasmic membrane transport systems. It may be that these components have particularly high affinities for iron and there is a subsequent loss of structure-activity relationships as all inositol phosphates yield iron readily to these systems.

It can be seen that *myo*-$InsP_6$-mediated iron transport provides an alternative mechanism for iron acquisition. Whilst the exact mechanism of action is unclear and may not necessarily confer a competitive advantage in the environment, it does

provide an alternative strategy for iron acquisition that reduces the metabolic demands created by siderophore biosynthesis.

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